Universidade de Aveiro Departamento de Biologia
Ano 2016

Sandra Sofia Cachulo Nunes

CARACTERIZAÇÃO, MICROPROPAGAÇÃO E PRESERVAÇÃO DE GENÓTIPOS DE *PINUS*

CHARACTERIZATION, MICROPROPAGATION AND PRESERVATION OF *PINUS* GENOTYPES

Universidade de Aveiro
Ano 2016

Departamento de Biologia

Sandra Sofia Cachulo Nunes

CARACTERIZAÇÃO, MICROPROPAGAÇÃO E PRESERVAÇÃO DE GENÓTIPOS DE *PINUS*

CHARACTERIZATION, MICROPROPAGATION AND PRESERVATION OF *PINUS* GENOTYPES

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Maria da Conceição Lopes Vieira dos Santos, Professora Catedrática da Faculdade de Ciências da Universidade do Porto, da Doutora Maria Celeste Pereira Dias, Investigadora do Pós-Doutoramento no CEF - Centro de Ecologia Funcional e no QOPNA – Unidade de Investigação de Química Orgânica, Produtos Naturais e Agroalimentares da Universidade de Aveiro e da Doutora Liliana Maria Bota Marum da empresa KLÓN, Innovative Technologies from cloning, S.A.

mais CENTRO Programa Operacional Regional do Centro
QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL PORTUGAL 2007.2013
UNIÃO EUROPEIA Fundo Europeu de Desenvolvimento Regional

Apoio financeiro do projecto NUP: CENTRO-07-0202-FEDER-018579, co-financiado pelo QREN, no âmbito do Mais Centro-Programa Operacional Regional do Centro, com a comparticipação do FEDER

Dedico esta tese às duas pessoas mais importantes da minha vida, que têm sido a minha rede de salvação, permitindo que arrisque sempre mais e mais alto, sem receio de cair... ***Aos meus pais***.

O júri / the jury

Presidente / President

Prof. Doutor Vitor Brás de Sequeira Amaral
professor catedrático do Departamento de Física da Universidade de Aveiro

Vogais / Members

Prof. Doutora Maria da Conceição Lopes Vieira dos Santos
professora catedrática da Faculdade de Ciências da Universidade do Porto (**orientadora**)

Prof. Doutor António José Brito Fonseca Mendes Calado
professor auxiliar do Departamento de Biologia da Universidade de Aveiro

Doutora Vanessa Isadora Tolentino Almeida Santos Pereira
Investigadora Sénior da empresa Klón – Innovative Technologies from Cloning, S.A. de Cantanhede

Doutora Sandra Isabel Marques Correia
Estagiária de Pós-Doutoramento da Universidade de Coimbra

Doutor José Miguel Pimenta de Oliveira
Estagiário de Pós-Doutoramento da Universidade de Aveiro

Agradecimentos / acknowledgements

This PhD Thesis resulted of a collaboration Project started in 2011 between the Laboratory of Biotechnology and Cytomics (Department of Biology, University of Aveiro) and the company KLÓN, Innovative Technologies from Cloning. So I would start with a word of thanks to KLÓN's administration, which enabled the realization of this thesis under the Project.

I would like to express my gratitude to my supervisors, Prof. Conceição Santos, Celeste Dias and Liliana Marum for giving me the necessary guidance, support and encouragement to reach my goals and for all the brainstorming sessions and good advices.

To my colleagues from KLÓN due to the companionship and the invaluable help that they gave me in the development of some of the tasks presented in this thesis. A special thanks to Nelson and Nélia for all the hours spent side by side in the flow chamber.

I wish to express my gratefulness to my colleagues from the Laboratory of Biotechnology and Cytomics that have been by my side giving me encouragement through these years. Diana, Glória, Joana, Cláudia and Bárbara couldn't have done it without you!

I must also thank my friends and family who supported me at every stage of this challenge. They are largely responsible for keeping my sanity along the way!

Finally, I would like to thank my life partner Rúben for his understanding through the years, for the words of strength and affection that gave me courage in times of crisis, and for the support he gave me principally during the thesis elaboration. You are my courage!

Palavras-chave

Pinus elliottii, Pinus elliottii x Pinus caribaea, coniferas, híbrido, micropropagação, embriogénese somática, criopreservação, crioprotector, armazenamento em arrefecimento lento, estabilidade genética, desempenho das plantas, fotossíntese, citometria de fluxo, programas de melhoramento.

Resumo

Pinus elliottii var. *elliottii* e o híbrido, *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis*, têm um grande valor económico, devido à sua elevada taxa de crescimento e produção de resina. Surge assim a necessidade de desenvolver estratégias de propagação mais eficientes desta espécie e do híbrido, mantendo as suas características. Este estudo tem como objetivos preservar / aumentar o banco de germoplasma de *Pinus*, fornecido pela empresa KLÓN, Innovative Technologies from Cloning, utilizando técnicas de micropropagação e de criopreservação, analisar possíveis alterações na estabilidade genética das plantas micropropagadas através de citometria de fluxo, estudar a taxa de sobrevivência de plantas, crescimento e desempenho fotossintético após aclimatização.

No Capítulo I foram abordados aspetos gerais da espécie e híbrido em estudo, assim como uma breve descrição dos programas de melhoramento em *Pinus* spp. e da contribuição que técnicas inovadoras de propagação *in vitro* podem dar levando a décadas de antecipação dos resultados dos respectivos programas de melhoramento. Foram também descritos alguns aspetos mais importantes relativos às diferentes técnicas de micropropagação e criopreservação existentes, apresentando sempre uma revisão do conhecimento atual sobre o uso destas técnicas no género *Pinus*. Para finalizar este capítulo, os objetivos de investigação desta Tese são apresentados.

O Capítulo II é dedicado à aplicação de uma metodologia de micropropagação (por proliferação de rebentos axilares) da espécie *Pinus elliottii* var. *elliottii*. Este capítulo foi dividido em duas secções. Na secção II.1 foi otimizado um protocolo para micropropagação em larga escala de *P. elliottii* desde a desinfecção e germinação de sementes para a produção de plântulas *in vitro*. Estas por sua vez são utilizadas como explantes para a indução de rebentos. Foram testadas várias condições para a indução, alongamento e enraizamento de rebentos, tendo sido estabelecido um protocolo que permite a produção de plântulas micropropagadas 20 a 22 semanas após germinação *in vitro*. Na secção II.2 foi realizada a caracterização genética e fisiológica de plantas de *P. elliottii* micropropagadas pela metodologia desenvolvida na secção anterior, em comparação com plantas provenientes de sementeira. O desempenho fisiológico das plantas foi avaliado pela determinação de diversos parâmetros relacionados com a fotossíntese e o metabolismo de carbono, tais como a fluorescência de clorofila *a*, o teor relativo em água, as trocas gasosas, o teor de pigmentos e de carbohidratos.

Resumo (cont.)

Por sua vez a caracterização genética foi realizada pela análise do conteúdo em DNA e nível de ploidia, e ainda as dinâmicas do ciclo célular, com recurso à citometria de fluxo. Os resultados obtidos indicam que o protocolo de micropropagação desenvolvido para *P. elliottii* não provoca alterações significativas tanto a nível fisiológico como genético nas plantas.

O Capítulo III centra-se na otimização de um processo de embriogénese somática para o híbrido *Pinus elliottii* var*. elliottii x Pinus caribaea* var. *hondurensis*, desde a iniciação até à regeneração de plantas produzidas a partir de embriões somáticos. Para a iniciação de culturas embriogénicas deste híbrido foram utilizados como explantes megagametófitos imaturos obtidos a partir de polinização aberta de cinco árvores *plus*. Para a otimização do processo foi avaliado o efeito do genótipo tanto na iniciação como na maturação, tal como o influência de diferentes formulações de meios basais e reguladores de crescimento nas diversas fases do processo. Ao longo do processo foi avaliada a estabilidade genética das massas embriogénicas com diferentes tempos de cultura, e no final das plântulas produzidas, em comparação com as agulhas das árvores-mãe, concluindo-se que foi desenvolvido um protocolo que permite a produção de plantas provenientes de embriões somáticos não tendo sido detectada variabilidade ao nível de conteúdo em DNA e nível de ploidia.

O Capítulo IV é dedicado à preservação do banco de germoplasma produzido para o híbrido em estudo. A criopreservação de massas embriogénicas é benéfica não só para a preservação de germoplasma durante o desenvolvimento de programas de melhoramento, como para evitar a perda do potencial embriogénico das massas. Para a otimização de um protocolo de criopreservação de massas embriogénicas pelo método de congelamento lento, foram testadas diferentes variações nos pré-tratamentos e na duração do passo de congelação lenta.

Os pré-tratamentos a que o tecido embriogénico foi submetido não influenciaram negativamente a capacidade de maturação das massas criopreservadas, apresentando-se a criopreservação até em alguns genótipos com um efeito benéfico. O protocolo otimizado permitiu a regeneração de plantas a partir de massas criopreservadas, para as quais se comprovou que o processo não provocou alterações genéticas, através da análise por citometria de fluxo de massas embriogénicas crio e não criopreservadas.

Finalmente, no Capítulo V são apresentadas as conclusões finais da Tese de Doutoramento, onde são realçados os avanços realizados como resultado desta tese nas metodologias de propagação e preservação para a espécie e híbrido em estudo. Neste capítulo são também apresentados os desafios futuros para a continuação da investigação nas áreas de propagação e preservação de *Pinus*.

Keywords

slash pine, *Pinus elliottii, Pinus elliottii x Pinus caribaea*, coniferous, hybrid, micropropagation, somatic embryogenesis, cryopreservation, cryoprotectant, slow cooling storage, genetic stability, plant performance, photosynthesis, flow cytometry, breeding programs.

Abstract

Slash Pine (*Pinus elliottii* var. *elliottii*) and the hybrid (*Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis*) have a great economic value due to their high growth ratio and resin production. Therefore, it is important to achieve a strategy to propagate this species and the hybrid more rapidly maintaining their characteristics. This project aims to preserve/enlarge the *Pinus* germplasm collection, provided by the company KLÓN, Innovative Technologies from Cloning, by micropropagation and cryopreservation techniques and analyze the putative changes (genetic stability) in micropropagated plants by flow cytometry, to study plant survival rates, growth and photosynthetic performance after acclimatization.

In Chapter I, general aspects of the species and hybrid under study are presented, as well as a brief description of the breeding programs in *Pinus* spp. and the contribution that innovative techniques of *in vitro* propagation can give, leading to decades of anticipation on the breeding programs results. It was also described some major aspects to the different micropropagation and cryopreservation techniques, always presenting a review of current knowledge on the use of these techniques in the genus *Pinus*. Finally, the research objectives of this thesis are presented.

Chapter II is dedicated to the application of a micropropagation protocol by proliferation of axillary shoots in the specie *Pinus elliottii* var. *elliottii*. This Chapter was divided in two sections. In section II.1 a protocol was optimized for large-scale *P. elliottii* micropropagation, which describes all the steps from disinfection and seed germination to the production of seedlings *in vitro*, which were used as explants for shoot induction. Various conditions for induction, shoot elongation and rooting were tested, and a protocol enabling the production of micropropagated plantlets 20 to 22 weeks after germination *in vitro* has been established. In section II.2 was performed the genetic and physiological characterization of *P.elliottii* plants micropropagated by the methodology developed in the previous section, in comparison with seedlings. The physiological performance of the plants was evaluated by determination of various parameters associated with photosynthesis and carbon metabolism, such as: chlorophyll a fluorescence; relative water content; gas exchange; pigment and carbohydrate contents. In turn, the genetic characterization was performed by analysis by flow cytometry of putative alterations in DNA content, ploidy level and in cell cycle dynamics. The results indicate that the developed micropropagation protocol for *P. elliottii* did not induce significative changes, both at physiological and genetic level, in the plants.

Abstract (cont.)

Chapter III focuses on the optimization of a somatic embryogenesis process for the hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis*, from the initiation to the plants regeneration produced from somatic embryos. For initiation of embryogenic cultures of this hybrid, immature megagametophytes obtained from five open-pollinated plus trees were used as explants. To optimize the process, the effect of genotype in both the initiation and maturation, as the influence of different formulations of basal media and growth regulators in the various stages of the process were evaluated. Throughout the process was assessed the genetic stability of embryogenic masses with different time in culture, and at the end of the produced emblings, in comparison with the mother-trees needles. This protocol allows the production of emblings from somatic embryos not having been detected variability in the DNA content and ploidy level.

Chapter IV is dedicated to the preservation of germplasm bank produced for the hybrid under study. Cryopreservation of embryogenic masses is beneficial not only for the preservation of germplasm during the breeding programs development, as well as to avoid the loss of the potential of the embryogenic masses. For the optimization of an embryogenic masses cryopreservation protocol using the slow freezing method, different variations were tested in pretreatments and in the duration of slow freezing step.

Pretreatments to which the embryogenic tissue was subjected, did not compromise the maturation capacity of cryopreserved masses. On the contrary, cryopreservation had in some genotypes a beneficial effect. The optimized protocol allowed the regeneration of plants from cryopreserved masses and the process did not induce major genetic changes (embryogenic masses cryo and non cryopreserved were analyzed by flow cytometric).

Finally, Chapter V presents the final conclusions of this PhD thesis, gathering the results of this thesis on the propagation and preservation methodologies for the species and hybrid in study and discussion this contribution to the state of art in this field. Future challenges for further research in these areas are presented in this Chapter.

ABBREVIATIONS

2,4-D - 2,4-dichlorophenoxyacetic acid

2iP - 2-isopentenyladenine

%DI - dispersion index

Φ_{PSII} - effective photochemical efficiency of PSII

A - CO_2 assimilation rate

ABA - abscisic acid

AC - activated charcoal

ASP - axillary shoots proliferation

BAP - 6-benzylaminopurine

Chl - Chlorophyll

CPPU - N-(2-Chloro-4-pyridyl)-N'-phenylurea

CSF - classical slow freezing method

CSO - clonal seed orchards

CV - coefficient of variation

DO - direct organogenesis

DW - dry weight

E - transpiration rate

ECL - embryogenic cell line

EM - embryogenic mass

FCM - Flow cytometry

FS - filter-sterilized

F_v/F_m - Maximum quantum efficiency of photosystem II (PSII)

FW - fresh weight

FWi - regrowth rate

GR - growth regulators

GS - genotypic selection

Hy - Hybrid *Pinus elliottii* var. *elliottii* x *Pinus caribaea* var. *hondurensis*

IAA - indole-3-acetic acid

IBA - indole-3-butyric acid

IO - indirect organogenesis

mDCR - modified DCR medium

mLV - modified Litvay's medium

MS - mass selection

mT - meta-topoline

MVF - multi-varietal forestry

NAA - α-naphthaleneacetic acid

NPQ - non-photochemical quenching

OP - open pollinated

PCH - *Pinus caribaea* var. *hondurensis*

PE - *Pinus elliottii* var. *elliottii*

PEG - polyethylene glycol

PGR - plant growth regulators

Pi - propidium iodide

PI - fluorescence intensity

PP - photoperiod

PPFD - photosynthetic photon flux density

PSII - photosystem II

qP - photochemical quenching

RAPD - randomly amplified polymorphic DNA

RH - relative humidity

RRS - reciprocal recurrent selection

RS - recurrent selection

RWC - relative water content

SCA - seed collection areas

Se - somatic embryos

SE - somatic embryogenesis

SPA - seed production areas

SSF - short slow freezing method

SSO - seedlings seed orchards

TDZ - thidiazuron

TSS - total soluble sugars

TW - turgid weight

WPB - Woody Plant Buffer

Z - zeatin

ZE - zygotic embryos

INDEX

CHAPTER I

GENERAL INTRODUCTION

Part of this chapter is in preparation to be submitted, as a review on *Pinus* micropropagation and preservation, to a SCI journal

Species Characterization

Forest trees are an integral part of human life, and a vital component of biodiversity. Conifers in particular are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products, like resin (Giri et al. 2004). Indeed conifers (cone-bearing trees) are the best-known and most economically important among gymnosperms, covering approximately 60% of the forested areas of the world (Ragonezi et al. 2010).

Conifers are considered living fossils, with an ancient evolutionary history, these trees survived to the continental drift, climate oscillations, the volcanism and the rapid spread of angiosperms (Williams 2009).

Conifers belong to the division of Pinophyta, also known as division Coniferophyta or Coniferae. The conifer designation is based on the fact that for most of the plants of this division the seeds occur in specialized structures, the strobilus, cone-shaped. This division comprises eight families, about 70 genera and 764 species including pines (*Pinus* spp.), spruces (*Picea* spp.), cowtail pine (*Cephalotaxus* spp.), cypress pine (*Callitris* spp.), firs (*Abies* spp.), larches (*Larix* spp.), bald cypresses (*Taxodium* spp.), yellowwood (*Podocarpus* spp.), yews (*Taxus* spp.), arbor vitae (*Thuja* spp.) and junipers (*Juniperus* spp.) (Figure 1) (Farjon 1999; Ragonezi et al. 2010).

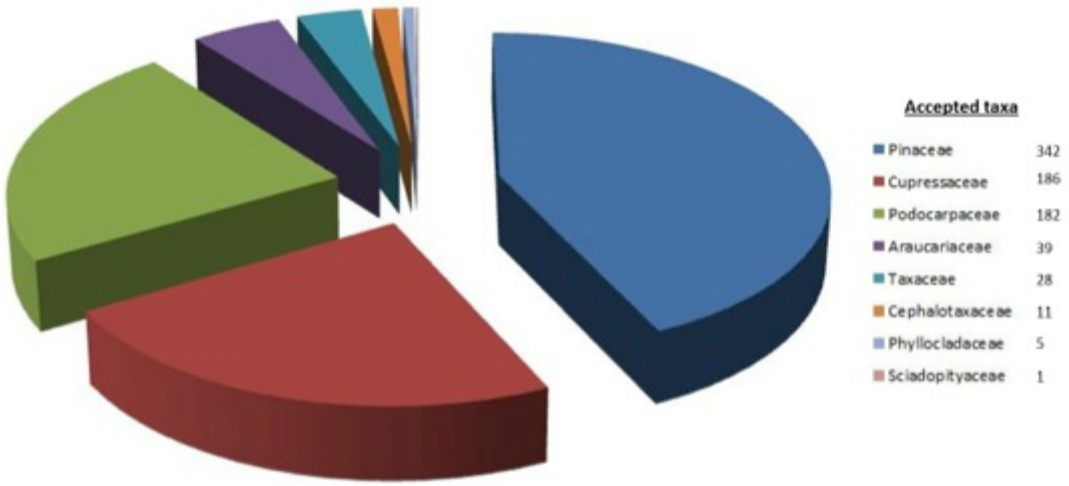

Figure 1 – Distribution of conifer species among the eight families (adapted from http://herbaria.plants.ox.ac.uk/bol/conifers).

The genus *Pinus*, with over 100 species, is the largest genus of conifers and the most widespread genus of trees in the Northern Hemisphere. Most classifications of the genus *Pinus* recognize two major lineages: subgenus *Strobus* (haploxylon or soft pines, with one fibrovascular bundle in the needle) and subgenus *Pinus* (diploxylon or hard pines, with two fibrovascular bundles in the needles). This division is consistent with data from wood anatomy and secondary chemistry, and is supported by molecular phylogenetic studies (Gernandt et al. 2005; Williams 2009).

The natural distribution of pines ranges from arctic and subarctic regions of Eurasia and North America south to subtropical and tropical (usually montane) regions of Central America and Asia. In our days, pines are also extensively planted in temperate regions of the Southern Hemisphere, mainly due to reforestation programs needed to lower the pressure on existing forests because of the increase on the global demand for several wood products (Giri et al. 2004).

Pines are being very popular in reforestation mostly because of the following characteristics (Medrado 2011):

- fast growing species;
- light softwood wood, ranging from white to yellowish, suitable for manufacturing high-strength paper for packaging, newsprint and other paper;
- their wood is also widely used in high-value carpentry items such as furniture, window frames, panelling, floors and roofing;
- the possibility of resin extraction on a commercial scale, in some species, which is an important source of turpentine;
- hardiness and tolerant of poor soils and relatively arid conditions, enabling planting on marginal soils for agriculture and thus add value to the land with the additional production of wood;
- ornamental value for afforestation and landscaping.

Depending on the production goal and on the geographic area, different *Pinus* species has been selected for plantation. Taking Brazil as study model, species of this genus were introduced in Brazil around 1936 (Resende 1999). Within the 2 million hectares of *Pinus* that have been planted in Brazil until 2013, with average productivities ranging between 18

and 28 m^3 ha^{-1} $year^{-1}$ (Relatório Anual IPEF 2013), over than 1 million was only with the species *Pinus elliottii* and *Pinus taeda* (Assis & Resende 2011).

The main species of the genus *Pinus,* which are being used for forestry in Brazil, are listed below with their respective uses and aptness regions (Resende 1999):

- Long fiber pulp, paper and sawn timber in subtropical climates: *Pinus elliottii, Pinus taeda.*
- Long fiber pulp, paper and sawn timber in tropical climates: *Pinus caribaea, Pinus oocarpa, Pinus tecunumanii, Pinus patula* (not in very hot regions and of high altitude), *Pinus maximinoi*.
- Long fiber pulp, paper and sawn timber, in regions of transition between tropical and subtropical climates: *Pinus tecunumanii, Pinus patula* and *Pinus maximinoi*.
- Resin production: *Pinus elliottii*.

In addition to the existing botanical species of *Pinus*, there is also a possibility of hybridization, as long as the crossings remain restricted to the same subgenera, being able to be crossed, e.g.: *P. caribaea hondurensis x P. caribaea bahamensis, P. caribaea x P. tecunumanii, P. oocarpa x P. patula or P. elliottii elliottii x P. caribaea hondurensis* (Assis & Resende 2011).

Pinus elliottii var. elliottii

Pinus elliottii var. elliottii (PE), commonly known as the slash pine, is one of the two varieties of the species *Pinus elliottii*, being the other named by *Pinus elliottii var. densa,* which occurs only in the southern half and Keys of Florida. This later variety differs from the other not only in geographical location, but also in seedling development and wood density (Tang et al. 2006).

Kingdom: Plantae
Division: Pinophyta
Class: Pinopsida
Family: Pinaceae
Genus *Pinus*
Subgenus: *Pinus*
Species: *Pinus elliottii*
Variety: *Pinus elliottii var. elliottii*

Figure 2 – Plantation of *P. elliottii var. elliottii* (adapted from: http://resinadepinus.blogspot.mx/2013/01/serie-pinus-e-resina-pinus-elliottii.html).

This pine (Figure 2) is native to the region of Southeastern United States, covering the states of Florida, South Carolina, Georgia, Alabama, Mississippi and Louisiana (Rodrigues 2006; Almeida 2011). This species is adapted to a warm and humid climate with wet summers and dryer falls and springs (Lohrey & Kossuth 1990).

PE is a fast growing species and can live about 200 years, has between 18 and 30 m high, straight and cylindrical stem with a diameter between 0.60 m and 0.80 m, the canopy is usually irregular with radius greater than 3.0 m (Newton et al. 1995; Anália 2010). The leaves or needles are sharp and thin, with a bright green colour and length between 18 and 24 cm. Flowering usually occurs in the spring. It starts producing seeds at the age of 15-20 years with good production every 2-3 years. It is one of the two southern pines used for naval stores and also one of the most frequently planted timber species in North America (Lohrey & Kossuth 1990).

PE is well prized by the timber industry because of its fast growth and excellent utility for pulp, lumber, and poles. This pine is also characterized by the high quality resin production (Burns et al. 1991; Newton et al. 2005).

The resin gum of pines is the basis of many products such as adhesives, printing inks, paints, varnishes, adhesives, welds, detergents, cosmetics, foods, synthetic rubbers, among many others. It is initially separated in two parts, the volatile fraction that is called turpentine and the solid one rosin. Turpentine is evaluated by his composition, where the ones that have a high content of pinene have better quality and are the most wanted by the

chemical industry that buys this product for subsequent conversion on pine oil, fragrance, and flavouring compounds between others (Rodrigues 2006).

Pinus radiata is the one that has a higher content of pinene on turpentine, more than 95%, but has a low yield of resin production. PE, has the second best quality of turpentine (almost 90% of pinene), but is the second one with the higher yield of resin production, being for this reason preferably planted and indicated for resin extraction on a commercial scale (Coppen & Hone 1995).

The hybrid *Pinus elliottii var. elliottii x Pinus caribaea var. hondurensis*

The first attempt to cross *Pinus elliottii var. elliottii* and *Pinus caribaea var. hondurensis* was performed in 1955 in the state of Queensland - Australia, aiming to develop a hybrid with higher quality than their parents, knowing that these two species have complementary characteristics (Dieters & Brawner 2007).

Briefly *Pinus caribaea var. hondurensis* (PCH) is the tropical pine with highest geographical distribution. Its rapid growth, high-quality wood that is hard and resistant and high yields of resin production make it a useful tree for timber, pulp, carton, paper and veneer production. For this reason, PCH has become one of the most important *Pinaceae* for forestry, planted in South America, West Africa and Australia for commercial purposes (David et al. 1995; Freitas et al. 2005). Compared with the PE, the PCH has a faster growth, with a stem with few branches and a more uniform wood. However, the PE has a straighter stem, more dense wood, being more resistant to high winds and more tolerant to poorly drained soils (Nikles 2000).

Figure 3 – Hybrid *Pinus elliottii* var. *elliottii* x *Pinus caribaea* var. *hondurensis* (source: Resisul Fortaleza, Lda.).

The first experimental plantations with the hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis* F1 (Hy-F1) were established in southeast Queensland and in Byfield in 1958. The hybrid showed superior growth, adaptation to a wide variety of places and stem straightness higher or similar in comparison to both parental species when grown in areas with poorly drained soils (Nikles 2000). Studies performed in the first plantations, the Hy-F1 showed wood quality characteristics very similar to those of their parents (Harding & Copley 2000).

This hybrid superiority appears to be derived from a complementary recombination of traits from the two parental species – growth rate and high yield of resin production from PCH (resin hardening similar to PCH), combined with wind-firmness, adaptability to wet sites, high wood-density and stem straightness of PE (Dieters & Brawner 2007).

Despite the good characteristics presented by the Hy-F1, its commercial deployment was hampered due to low viability of the seeds and the difficulty of vegetative propagation of their seedlings (Nikles & Robinson 1989). In the mid 1980s attempts have been started to self-hybridization of Hy-F1, which resulted on the hybrid PE x PCH F2 (Hy-F2), that

showed to be an alternative to overcome the problems of viability of seeds and seedlings of the former hybrid (Figure 2) (Nikles 2000).

Breeding programs for Pinus spp.

As the human population grows, the demand for wood and the vast array of products made from it increases, making pine trees among the most valuable of commercial crops produced around the globe (Giri et al. 2004). Like it was established before, some pine trees, like PE, are not only a source of wood, but also a source of other value product, the resin.

Taking into account that these trees will take many years to give any return on the initial investment, such as in the case of reforestation aimed at producing wood and resin, it is important that the producer invests in seeds with high genetic quality, which can generate a plantation of fast-growing trees and high wood and resin quality. Also, the trees must be resistant to pests, diseases or adverse weather (Higa 2002). In this context, tree breeding programs are an essential tool for the achievement of the commercial success in forestry.

Large-scale tree breeding programs began in the 1950s, being PE one of the species that started to be improved at this time (Zobel & Talbert 1984). The overall objective of the breeding programs is the modification of the genetic heritage of a plant species to acquire the characteristics sought by the improver (Foelkel 2011). Genetic improvement is achieved by the existence of tremendous genetic variability within most tree species, allowing controlled crosses to obtain new improved genotypes. The improvement is obtained by recombination of genetic variation within and between populations of the genus through crossing or biotechnology, obtaining new varieties or improved genomes (White & Byram 2004).

There are several steps involved in the production of seeds and seedlings of genetically improved *Pinus*, aiming to obtain improvements in the following characteristics: forestry productivity, resistance to pests and diseases, stem form and development (volume, height and diameter), wood and resin quality, yield of resin production and target climate adaptation. In these programs, maintaining genetic variability is imperative for obtaining genetic gains at long term (Furlan et al. 2007).

A breeding program involving, at once, all the characteristics described above would be extremely complex. So these programs are established in stages, wherein one or two features are select for improvement. For example when it is desired to obtain genetic gains in the production of wood and resin, will be more advisable to conduct two separate programs, wherein one it will be improve the stem form and development, and in the other the quantity and quality of the resin (Fonseca & Kageyama 1978).

Breeding strategies

One of the oldest and historically most successful schemes to achieve the characteristics described above in *Pinus* species is the method of genetic improvement referred to as recurrent selection (RS). Recurrent selection uses repeated cycles of breeding aimed at the gradual and cumulative improvement of a few traits in a population (White & Byram 2004).

The first step of a breeding program using the RS method is the selection of the individuals that will be on the basis for the improvement. This step is of high relevance within the breeding program because it is about the selected material that will focus future work program. So to start the selection, the objectives of the improvement must be very clear and established (Fonseca & Kageyama 1978). There are two methods for this selection: Mass selection (MS) or Genotypic selection (GS) (Foelkel 2011).

Mass selection of individuals is based solely on phenotypic characteristics observed on a base population (Briggs & Knowles 1967). Seeds are collected from the phenotypically-superior individuals to establish new populations. The superior selected trees for seed production are at most 10 or 20% from the original population (Fonseca & Kageyama 1978). In this way, seed collection areas (SCA) or seed production areas (SPA) can be established (Foelkel 2011). On the SCA the inferior individuals are not eliminated from the area allowing their crossing with the selected ones. The selection is performed on the female side since there's no control of the pollinating trees (male side). Fonseca and Kageyama (1978), giving as an example the improvement of resin production in PE, attribute to this method a genetic gain of 17%. To establish a SPA all the inferior trees are eliminated from the population remaining only the phenotypically-superior individuals. In this way the selection is performed on both female and male sides with a genetic gain of

35% for the resin production improvement on PE (Fonseca & Kageyama 1978; Foelkel 2011).

RS-MS is rarely used for modern tree breeding programs, because it is less efficient at achieving genetic gains than forms of recurrent selection that incorporate genetic testing (Namkoong et al. 1988). However, this method is still used on the initial phases of the breeding programs or to obtain short-term improved seeds (Foelkel 2011).

The genotypic selection is based on progeny tests where the breeding values of parents are evaluated by tracking the performance of their offspring (White & Byram 2004). These tests measure the parents capacity to transmit the genes that result on the desirable phenotype to their descendants. Progeny tests are needed to establish seedlings seed orchards (SSO) and clonal seed orchards (CSO), on these orchards, improved seeds can be obtained from free or controlled pollinations. SSO are plantations of seedlings from selected trees after the progeny tests and are used mostly when the vegetative propagation is difficult for the target species. CSO are obtained through vegetative propagation from the genetically superior trees (Fonseca & Kageyama 1978).

Genetic testing greatly increases the genetic gain above that possible from mass selection. This is especially true for traits with low heritabilities which, unfortunately, account for most economically-important traits of conifers (White & Byram 2004). Taking again as example the improvement of resin production in PE Fonseca and Kageyama (1978) achieved a genetic gain of 59.4% on 1st generation CSO, the same that was achieved on SSO. But this genetic gain increased significantly on 2nd generation CSO reaching a value of 152%.

Each cycle of breeding on RS-GS is composed by the subsequent steps: obtaining progenies; establishment of progeny tests; selection of superior progenies; recombination inter or intra species between the selected progenies (Aguiar et al. 2011).

According to Resende and Barbosa (2005), the principal strategy to improve interspecies crossings is reciprocal recurrent selection (RRS), and is based on the prior selection within each specie, with complementary characteristics, and crossing by controlled pollination of superior individuals to produce hybrids. This approach has been extensively used because it offers the potential to expand the area over which a *Pinus* specie may be successfully deployed, through broaden adaptability, complementary combination of economically

important traits, and the potential to breed for improved hybrid performance (Dieters & Nikles 1997). Example of this is the extensively hybridization that has been undertaken, since the mid 50's, in Queensland among several *Pinus* species with the aim of developing high-yielding varieties with suitable wood properties and adapted to sites of industrial plantations (Nikles 2000). Like it was described before the hybrid between PE and PCH arose from these works.

In 2000, the company Pinus Brasil implemented experimental plots with the hybrid *P. elliottii x P. caribaea*. Studies conducted so far by this company on the development of the hybrid compared with other faithful samples of pure plantations of *P. elliottii* and *P. taeda*, revealed that the 5.6-year-old hybrid had an annual average increase of 44.8m³ / ha / year, higher than the values obtained for *P. elliottii* (18.8m³ / ha / year) and *P. taeda* (28.5m³ / ha / year). The precocity and higher productivity per hectare, lower operating costs and, consequently, faster return of the investment, support the cultivation of this hybrid as a potential generator of resources and investment in the region (Pinus Brasil 2007).

Breeding programs for PE started on the 50's in the southern USA with the objectives of increase resistance to *Cronartium fusiforme* (rust), volume yield, and stem straightness. These programs started with massive selection to obtain phenotypically-superior individuals, followed by vegetative propagation and establishment of progeny tests to select breeding populations and implement CSO (White & Byram 2004). In Brazil the first breeding attempts for PE were made also in mid 50's, but was in 1967 that the first large-scale breeding programs have started in order to obtain quality raw material for the paper and pulp industries (Foelkel 2011). Later, because of the high quality of its resin, the breeding programs for PE began to focus on the improvement of the resin production yield, however the efforts for these improvement are still scarce (Shimizu & Spir 1999).

Vegetative propagation on breeding programs

The ability to full regeneration, forming complete individuals from a single cell or any part of the individual tissue with living cells is called totipotency. This plant's characteristic allows its vegetative propagation, also known as cloning, based solely on mitosis. This happens because somatic cells, capable of cellular differentiation, are present in many plant tissues. The totipotency does not manifest in the same way in all plant species, being more

or less intensive in different types of cells and activated by different conditions, depending on the species. This exceptional regeneration capacity also allows to join a part of an individual as part of another, by placing the two parts in close contact, so that the tissue regeneration join them to form a single plant (Floriano 2004b). This form of asexual reproduction allows the production of large quantities of selected plants with the same characteristics from the mother.

These techniques have a great importance on breeding programs, like it was described before, which are on the basis of the establishment of CSO, an essential tool for trees improvement (Pascoe 2002). Vegetative propagation allows also the conservation of pine genotypes, as it promotes the plants physiological rejuvenation, and the germplasm rescue, regardless of the availability of seed (Aguiar et al. 2011).

Despite all the advantages of *Pinus* species vegetative propagation, it should be taken particular attention to the risk of narrowing of the genetic base of clonal plantations that occurs when using a small number of clones, which could lead to non-occurrence of additional genetic gains after the first generation of selection (Brune 1982).

The main factors affecting the vegetative propagation of plants are: the propagules maturation / juvenility, mineral nutrition of the mother plant, growth regulators, luminosity, temperature, humidity, propagation method, among others (Wendling 2003).

There are several methods to achieve vegetative propagation, which are divided into four groups: Cuttings; Grafting; Layering; Micropropagation (Figure 4). The use of each method varies with the desired production goals as well as the species, time of year, the type and amount of available material, and environmental conditions (Faria 2012).

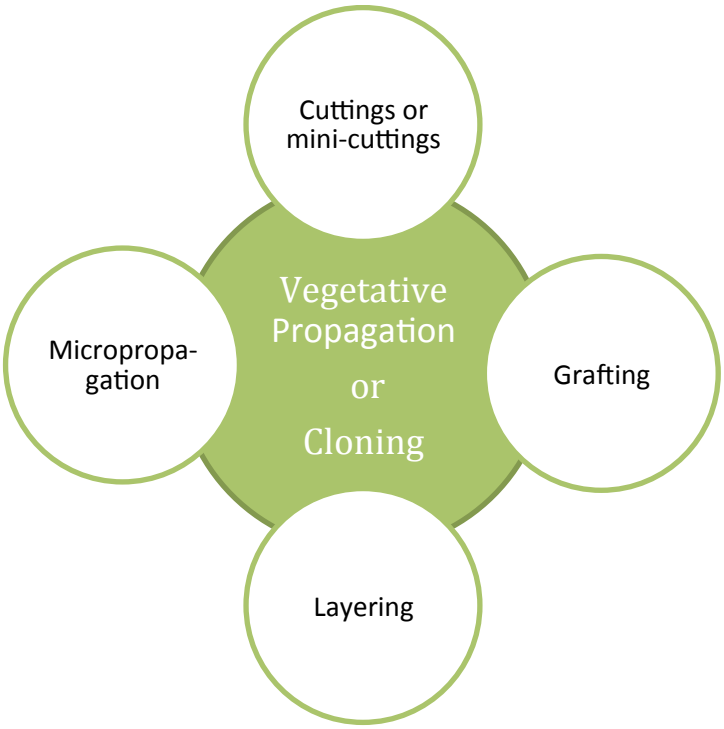

Figure 4 – Methods of vegetative propagation on forest plants.

Vegetative propagation by **cuttings** is the most used technique in forests for producing selected plants on a large scale. In this method 4 phases can be distinguished, starting with the production of shoots, followed by cutting and preparation of the growth medium, third rooting and four the acclimatization of the plants propagated. The most important phases are the shoots production and rooting, because they can limit the amount of cuttings produced (Floriano 2004b). For pine trees, vegetative propagation by cuttings has been studied for several decades. Despite the general trend in companies to adopt and gradually increase the use of rooted cuttings, especially for the spread of eucalyptus in Brazil, the use of rooted cutting of pine is restricted. This fact is due mainly to the difficulty of rooting and recovery of physiological plant age. In this case, the use of juvenile material is required to successful rooting (Aguiar et al. 2013).

To overcome the problem of rooting, vegetative propagation of pine species by **mini-cuttings** has been used in various countries (Higashi & Silveira 2004; Andrejow 2006). Mini-cuttings presents a number of advantages over traditional rooting of cuttings, as the operational benefits (involvement of hand labor in the preparation of cuttings and rooting without hormones application), higher degree of shoots juvenility (increasing the degree of initiation and root growth, resulting in increase on plant quality), and the decrease of expenses on the deployment, cultivation, irrigation, and fertilization management (Assis

1997). However, this method presents some disadvantages compared to conventional cuttings: mini-cuttings are more sensitive to environmental conditions, require an elaborate production schedule, skilled labor and more control especially regarding nutritional and water availability because of the higher sensitivity of mini-cuttings (Xavier 2002). In this method, the vegetative propagules are obtained by the apical pruning of a rooted cutting or seedlings forming the mother plants. The mother plants can be kept in organic substrate with timely fertigation, or in a hydroponic system where washed sand is often used as support. This procedure leads to the development of new shoots that are collected and cultured in the greenhouse for rooting. The production rate depends on the time of year, the clone / species, the nutritional conditions, among others (Andrejow 2006).

Grafting is made by junction between two parts of plants of the same family or genus: the graft - part of the plant to be multiplied, and the rootstock or horse - part of another plant that receives the graft). Depending on the species and environmental conditions, the graft is considered viable 20 to 40 days after grafting. The branches of the horse should be pruned later to promote apical dominance of the graft (Faria 2012).

The main objectives of grafting are the attainment of higher vigour and productivity, of resistance to disease and pests, plant postage modification, restoration of individuals already in production that are losing vitality, creating varieties, premature flowering and fruiting, better quality and greater production of seeds (Gomes 1990). Grafting in pine is most used for research purposes and for the development of CSO. Generally, propagules for grafting are collected from the third upper branches of the tree canopy. The period recommended for the *Pinus* grafting comprises the late spring to early summer (Aguiar et al. 2013).

Layering is the process of vegetative propagation where a branch of a plant is dipped in the soil to rooting, being then separated from the mother plant and becoming an independent plant, a process that can take up to 3 months (Farrar & McJannet 1959). It is the vegetative propagation method that has the highest percentage of rooting, although low yield (Simão 1998). Layering can be made either by bending the branch to the ground as by the involvement of a branch with soil, in which case is called air layering and is the technique used on *Pinus* (e.g. Farrar & McJannet 1959; Arya & Haque 1982). It is recommended the early spring period for this operation, preferably using branches with

less than a year (Floriano 2004b; Faria 2012). Currently this technique is rarely used in forest species.

The vegetative propagation methods described above frequently have problems of loss of juvenility and supply of tissue. To overcome these problems, *in vitro* vegetative propagation techniques, also known as **micropropagation,** have being developed (Smith et al. 1994). Micropropagation promotes plants production from different organs that may be, buds, embryos, hypocotyl segments or a single cell in a sterile culture. These techniques offers great possibilities for commercial plant propagation, making possible to obtain large numbers of individuals from a few mother plants, in a short time and reduced laboratory area. Can also assist in breeding programs, enabling decades of anticipation on the final results. However, the use of micropropagation in the commercial production of forest species is rarely justified at the technical and economic levels (Wendling 2003). That's why it has been heavily invested in the development of micropropagation techniques for a variety of forest species. In the next section of this chapter, an overview is given on the existing types of micropropagation and the advances made in this area for pine trees.

In vitro approaches for Pinus spp. propagation

Micropropagation is by definition an *in vitro* vegetative propagation process in plant tissue culture (Hartmann & Kester 1975; Torres et al. 2000). It's a way to rapid production of thousands of clones of a plant from a single somatic cell or a small piece of plant tissue (explants) (Andrade 2002).

The explants are a mixture of cells in different states: physiological, biochemical and developmental. Accordingly, it is expected that exposure of these explants to *in vitro* environment encourages diverse responses in different cell types, so that only few cells respond to *in vitro* explant culture conditions, leading to regeneration of a new individual (Mantell et al. 1994). This ability that a cell or group of cells has to respond to specific signals, like growth regulators substances, light, temperature, etc., leading to a development process to form a new plant is called competence (Torres et al. 2000).

A successful micropropagation technique is strongly influenced by the genotype, chronological/physiological age of the donor plant, in other words the explant source and

by the culture conditions (Andrade 2002; Rodríguez et al. 2005; Valledor et al. 2007) . The success of *in vitro* culture initiation and regeneration depends on the correct balance in the establishment of all these factors.

The explant source is an important factor to consider for the success of *in vitro* regeneration, because the regenerative capacity depends on the maturity, physiological state and tissue used. Generally, young growing tissues are used as explant source, but at the end the explant selection depends on the final aim of the program (Aitken et al. 1981). Indeed there are different kinds of explants used in *Pinus* spp. micropropagation depending on the researcher´s objective, like embryos, cotyledons or apical buds and branch tips from mature tissues (Valledor et al. 2007). Such is also valid for the selection of genotypes. Interestingly, varieties of the same species respond differently to the growing conditions, although some authors consider that all species are able to respond to *in vitro* culture conditions if the appropriate combination is used of all factors that affect the *in vitro* regeneration (Mantell et al. 1994; Andrade 2002).

The culture conditions, especially the culture medium, are decisive for the success of *in vitro* regeneration. Usually a variety of micro and macronutrients, carbon sources, vitamins and growth regulators are tested in order to find the best combination for the propagation of genotype under study. The appropriate combination of these components, associated with other culture conditions such as light (intensity, quality, photoperiod), temperature and the culture vessel (size and permeability to gas exchange) is the base of the plant tissue culture technology (Kerbauy 1997).

Micropropagation techniques include the culture of apical and nodal segments promoting axillary shoots proliferation, the formation of adventitious buds on explants by direct or indirect organogenesis and somatic embryogenesis (Torres et al. 2000). Deberg and Maene (1981) propose a scheme to describe the micropropagation that is divided into five phases, and that in some way can be applied to all micropropagation techniques. The donor plant preparation is called the zero phase, which can be achieved for example by the treatment with hormones, disinfectants or fungicides, or by another pretreatments like loss of apical dominance by pruning the apex and shading to promote the stem elongation. Phase one is *in vitro* establishment and initiation where it comes to the explant selection and disinfection, and inoculation on culture medium on the selected ambient conditions. The

next phase is the multiplication for induction of new shoots, which for some techniques includes induction of calli from which adventitious organs developed (organogenesis). After induction, the shoots elongation is normally needed before the phase four of rooting, which is followed by the acclimatization.

In pine species, several studies on micropropagation have been reported in the literature using the different micropropagation techniques, most of these reports showing success on the achievement of micropropagated plantlets (for summary see Table 1).

Table 1 - Summary of micropropagation induction for *Pinus* species. ASP - axillary shoots proliferation; DO – direct organogenesis; IO - indirect organogenesis; PP – photoperiod; nd – not defined; ZE – zygotic embryos.

Species	*Method*	*Explant type*	*Basal Medium*	*Culture conditions*	*Carbon source*	*PGR*	*Response*	*Acclimated plantlets*	*Reference*
P. ayacahuite	DO	Cotyledons	MCM	25ºC, 16h PP	≈ 3% sucrose	BAP	Rooted shoots	Yes	Saborio et al. 1997
P. caribaea	DO	Mature ZE	MSM (modified MS)	25 - 27 ºC, 16h PP	≈ 3% sucrose	BAP	Rooted shoots	No	Go et al. 1993
	DO	Mature ZE	MSM (modified MS)	25 - 27 ºC, 16h PP	3% sucrose	BAP	Rooted shoots	Yes	Halos & Go 1993
P. elliottii	ASP	Seedling apices	GD	24ºC, 16h PP	nd	BAP	Shoots	No	Burns et al. 1991
	DO	Cotyledons	BL	24ºC, 24h PP	2% sucrose	BAP	Rooted shoots	No	Bronson & Dixon 1991
	IO	Mature ZE	B5, SH or TE	Dark	3% sucrose	NAA, 2,4-D and 2iP	Rooted shoots (low rates of rooting)	Yes	Tang et al. 2006
	IO	Mature ZE	BMS, SH or TE	23ºC, Dark	3% sucrose	NAA, 2,4-D and 2iP	Rooted shoots (low rates of rooting)	Yes	Tang & Newton 2007
P. elliottii × *P. caribaea*	ASP	Tender stem segments	DCR	nd	3% sucrose	BAP and NAA	Rooted shoots	nd	Lv & Huang 2012
P. kesiya	ASP	Seedling apices	MS	25ºC, 16h PP	nd	Kinetin	Rooted shoots	nd	Nandwani et al. 2001
P. massoniana	ASP	Seedling apices	GD	25ºC, 16h PP	3% sucrose	BAP and NAA	Rooted shoots	Yes	Zhu et al. 2010
P. nigra	DO	Cotyledons	½ MS	25ºC, 16h PP	3% sucrose	BAP and NAA	Rooted shoots	Yes	López et al. 1996
P. roxburghii	ASP	Seedling apices	MS	25ºC, 16h PP	3% sucrose	BAP	Rooted shoots	Yes	Kalia et al. 2007

Species	*Method*	*Explant type*	*Basal Medium*	*Culture conditions*	*Carbon source*	*PGR*	*Response*	*Acclimated plantlets*	*Reference*
P. pinaster	DO	Cotyledons	GMD	25ºC, 16h PP	3% sucrose	BAP	Rooted shoots	Yes	Calixto & Pais 1997
	ASP	Seedling apices	WV	25ºC, 16h PP	3% sucrose	BAP and NAA	Rooted shoots	Yes	Azevedo et al. 2001
	ASP	Bud segments; Seedling apices	GD	23ºC, 16h PP	3% sucrose	BAP; BAP and NAA	Shoots; Rooted shoots	No; yes	Tereso et al. 2006
	ASP	Bud segments	DCR	23ºC, 16h PP	3% sucrose	BAP	Rooted shoots	Yes	De Diego et al. 2008
	DO	Cotyledons	TE	Dark	3% sucrose	BAP	Rooted shoots	Yes	Alvarez et al. 2009
	ASP; DO	Embryo axes; cotyledons	DCR	PP; dark	3% sucrose	TDZ	Rooted shoots (low rates of rooting)	Yes	Humánez et al. 2011
	DO	Mature ZE	½ DCR	22ºC, 16h PP	3% sucrose	mT	Rooted shoots	Yes	De Diego et al. 2011
P. pinea	DO	Cotyledons	½ LP	25ºC, 16h PP	3% sucrose	BAP	Rooted shoots	Yes	Alonso et al. 2006
	ASP	Bud segments	LP	22ºC, 16h PP	3% sucrose	TDZ	Rooted shoots (low rates of rooting)	Yes	Cortizo et al. 2009
P. radiata	DO	Mature ZE	LP	21ºC, 16h PP	3% sucrose	BAP or Z	Rooted shoots	Yes	Montalban et al. 2011
P. sylvestris	ASP	Seedling apices	1/8 MS	26ºC, 16h PP	3% sucrose	BAP	Rooted shoots	nd	Žel et al. 1988
	ASP	Bud segments	DCR or WP	22ºC, 16h PP	3% sucrose	mT	shoots	nd	De Diego et al. 2010
P. taeda	ASP	Apical shoots and nodal segments	WV5	19°C(night) and 28°C(day), 16h PP	3% sucrose	BAP	Rooted shoots	Yes	Oliveira et al. 2012
P. wallichiana	DO	Mature ZE	½ DCR	23ºC, 16h PP	2% sucrose	BAP and TDZ	Rooted shoots	Yes	Mathur & Nadgauda 1999

Axillary shoots proliferation

Micropropagation from axillary buds is a simpler technique compared to others (eg. organogenesis and somatic embryogenesis), which have been widely used for forest trees. This technique, in addition to using preformed meristems, it allows greater genetic stability, less somaclonal variation and also avoids using high concentrations of cytokinins for the development of axillary buds (Kalia et al. 2007; Oliveira et al. 2012).

Explants type and disinfection

Axillary shoots proliferation has been reported as successful in numerous *Pinus* species using different types of explants, being the apices from seedlings growing *in vitro* one of the most popular explant for axillary shoots proliferation (Azevedo et al. 2001; Tereso et al. 2006; Kalia et al. 2007; Zhu et al. 2010). The development of micropropagation protocols based on this type of explant is quite useful for the propagation of genotypes from controlled crosses in breeding programs, since there is usually a shortage of seeds, therefore they need to be propagated asexually. Seed surface disinfection, for *in vitro* germination, is a relatively simple and efficient process for the removal of exogenous contaminants, usually made with protocols based on hypochlorite solutions (Žel et al. 1988; Calixto & Pais 1997; Saborio et al. 1997; Tereso et al. 2006; Valledor et al. 2007; Humánez et al. 2011) or hydrogen peroxide solutions (López et al. 1996; Alonso et al. 2006; Alvarez et al. 2009; De Diego et al. 2011). In some cases, the use of mercuric chloride is also common (Mathur & Nadgauda 1999; Nandwani et al. 2001; Kalia et al. 2007), however due to its high toxicity it is becoming less udes. The addition of a few drops of detergent to the disinfectant solution, and a step of disinfection with ethanol are often performed to complete the protocols. After disinfection usually the seed coats are removed under sterile conditions and the megagametophytes placed on the germination culture medium (Zhu et al. 2010).

Micropropagation of adult trees can be initiated with different tissues, apical buds (De Diego et al. 2008; Cortizo et al. 2009; De Diego et al. 2010) or branch tips (Lv & Huang 2012), usually collected from vigorous trees. However mature tissues from field develop a higher contamination degree and even endogenous contaminants, what makes this type of

tissues more recalcitrant to disinfection protocols. Although the same disinfectants are used, usually the infection rates are higher for these tissues, which is why some disinfection protocols include an immersion step in fungicide (Valledor et al. 2007).

Axillary shoots induction and elongation

Axillary shoot induction is the more studied and documented stage, where several culture media have been tested in order to achieved the most appropriated growth conditions for each species, combining different basal media and growth regulators, mostly cytokinins. For *Pinus* species different media can be highlighted, e.g. MS (Murashige & Skoog 1962), GD (Gresshoff & Doy 1972), DCR (Gupta & Durzan 1985) and WV5 (Coke 1996). Lv and Huang (2012) have compared DCR with MS and GD to axillary shoot induction on the hybrid *Pinus elliottii* × *Pinus caribaea,* achieving better results with DCR. On the other hand, Oliveira and co-authors (2012), for *Pinus taeda*, tested MS versus WV5 for the same purpose, selecting WV5. For *Pinus pinaster,* Azevedo and co-authors (2001) selected a Westvaco medium over GD. The main difference between these culture media is the nitrogen availability, where WV5 and DCR have lower amount of nitrogen than GD and MS culture media. Oliveira et al. (2012) had referred that high concentrations of N in culture medium formulations may have a toxic effect in some species.

Cytokinins are essentials for axillary shoot induction since they are responsible for cell division in plants. For *Pinus* spp. the use of 6-benzylaminopurine (BAP) to the induction phase is the most reported, but others cytokinins have been tested with success, like thidiazuron (TDZ), kinetin and meta-topoline (mT) (see Table 1). Zhu et al. (2010) find that the addition of the auxin α-naphthyl acetic acid (NAA), improved the number of shoots per explant, and other authors have also reported protocols with this methodology for *Pinus* axillary shoot proliferation (Azevedo et al. 2001; Tereso et al. 2006; Lv & Huang 2012).

The addition of sucrose, as carbohydrate source, to the culture medium appears to be a general choice on published works for *Pinus* spp. (see Table 1). It is generally accepted that plants under *in vitro* conditions grow better on media containing the saccharides that is transported at long distance and easily metabolized by the plant. As sucrose is the

saccharide prevailing in the phloem sap of most plant species, it is the most commonly used carbohydrate for the support of *in vitro* cultures (Lipavská & Konrádová 2004).

After the induction stage it is common to perform shoot elongation in a hormone-free medium. In most of the protocols activated charcoal is also added in order to adsorb the hormones previously added (Azevedo et al. 2001; Tereso et al. 2006; Kalia et al. 2007; De Diego et al. 2008; Cortizo et al. 2009).

Rooting and Acclimatization

Rooting and acclimatization of shoots remain the bottleneck of the micropropagation process reducing the possibilities of applying this technique on a large scale. Rooting depends on the genotype and physiological condition at the time of root induction, and the acclimatization is hampered by the plantlets morphological anomalies, such as non-functional stomata, and physiological anomalies, such as a decrease in photosynthesis caused by the heterotrophic conditions of typical *in vitro* propagation (Zavattieri et al. 2009; Muniz et al. 2013).

However several rooting methodologies has been described for *Pinus* with success in many species like for exemple *P. roxburghii* (Kalia et al. 2007), *P. massoniana* (Zhu et al. 2010), *P. pinaster* (De Diego et al. 2011), *P. nigra* (López et al. 1996) and *P. pinea* (Alonso et al. 2006).

Usually the use of auxins is required to achieve micropropagated shoots rooting, being indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA) the most used for pine species. *Pinus* rooting can be promoted through a high concentration pulse treatment or using low concentration of auxin with higher exposition time (Valledor et al. 2007). For example, Calixto and Pais (1997) promoted rooting of *P. pinaster* by exposing shoots to high concentration of IBA (393,6μM) (auxin pulse) for a short period of time (24 hours) followed by a transfer to a mixture of peat and perlite. In turn, Kalia et al. (2007), for *Pinus roxburghii,* have achieved 70,83% rooted shoots using smaller concentrations of auxin (5 μM of NAA) in the culture medium during 15 to 20 days.

For the *Pinus* species studied in the present thesis, rooting protocols have been reported using low auxin concentration with at least 6 weeks of exposure. For the hybrid *Pinus*

elliottii × *Pinus caribaea*, more than 50% of rooting was obtained by Lv and Huang (2012) when shoots were inoculated on a culture medium with 10,75 μM of NAA for 40 days. Tang and co-authors (2006) achieved in *Pinus elliottii* rooted shoots by the exposure to 0,01 μM of IBA and indole-3-acetic acid (IAA) also for 6 weeks.

In alternative, some researchers promoted rooting using auxin in combination with a cytokinin. For example, Oliveira et al. (2012) used NAA (2,69 μM) and BAP (0,44 μM), during 12 days, for root induction on shoots of *Pinus taeda,* with a success rate of almost 50%. According to these authors, the cytokinin present in the rooting medium can prevent apical necrosis.

The maintenance of shoots in the dark during root induction seems to favour rooting, as described for *Pinus pinaster* (Calixto & Pais 1997; Humánez et al. 2011), *Pinus pinea* (Alonso et al. 2006; Cortizo et al. 2009) and the hybrid *Pinus elliottii* × *Pinus caribaea* (Lv & Huang 2012).

When the root induction is made through prolonged hormone exposure, usually it is followed by a root elongation step, which is performed at hormone-free media, frequently added with activated charcoal for the same purpose that it is used at the shoot elongation stage (Saborio et al. 1997; Azevedo et al. 2001).

It has been reported that shoots of *Pinus pinaster* maintained on elongation medium for 5-6 months, without subculturing, formed roots spontaneously (Calixto & Pais 1997). This fact can be correlated with the decay of nutrients in the medium, so many authors choose to use culture media with half-strength or less at elongation stage (López et al. 1996; Kalia et al. 2007; Oliveira et al. 2012; Lv & Huang 2012).

Acclimatization is the last and one of the most critical steps of micropropagation consisting of one of the limiting factors for the production of plantlets through micropropagation on a commercial scale, especially when it comes to species of *Pinus*, which do not always had a high percentage of survival (Oliveira 2011).

Roots formed *in vitro* often aren't functional due to their disconnection to the vascular system. Vascular connection between the stem and root is of great importance for water conduction and plant survival after transplantation. Some plants can develop this connection after a while, but others will suffer from this lack of connection during the

acclimatization process (Oliveira 2011). Moreover, during *in vitro* conditions, plants grow in a culture medium with a large concentration of sugar, under specific environment conditions of high relative humidity (>90%), low CO_2 concentration and low light intensity. These special conditions result in a formation of plants with morphology, anatomy and physiology poorly developed, such as undeveloped cuticle, open stomata and thin leaves photosynthetically not very active. Once transferred to *ex vitro* conditions, the plants are very susceptible to several stresses, such extra water loss, since they have not developed adequate features required to survive in the new environment (*in vivo*) (Dias et al. 2011; Dias et al. 2013).

So the acclimatization process should be done gradually for plants to adapt to *in vivo* climatic conditions. In *Pinus* the most described process involves the rooted shoot transference to a mixture of peat and perlite or vermiculite and acclimatization in a greenhouse or climatic chamber under controlled conditions. The process is started with high relative humidity close to 100%, and gradually reduced to values of 70-60% over 2 to 4 weeks. After the period of acclimatization, the plants can be transferred to ambient conditions (Azevedo et al. 2001; Tereso et al. 2006; De Diego et al. 2008; Cortizo et al. 2009; Humánez et al. 2011).

Adventitious shoots proliferation - Organogenesis

As mentioned before other potential micropropagation technique involves the regeneration of shoots by organogenesis. Organogenesis is a path of development in which plant organs (shoots, roots), or both are induced to differentiate from one or more cells of an explant. The organogenesis can be direct or indirect. In the direct organogenesis, also called of adventitia, plant organ formation is induced and develops directly from an explant, that is, without passing through an initial phase of callus. In indirect organogenesis, there is an initial phase of proliferation and growth of callus, followed by induction of shoots and roots and development of these tissues (Andrade 2002; Zavattieri et al. 2009).

Propagation techniques for pine species through organogenesis approaches have shown great versatility for a range of applications, including arresting of juvenile growth via cool storage and more recently developed cryogenic methods (Hargreaves & Menzies 2007).

Explants type and in vitro establishment

The most common explants to induce organogenesis on *Pinus* spp. come from mature zygotic embryos, either the embryo itself or its cotyledons (see Table 1), isolated from seed megagametophytes .

Most likely, mature embryos could be the most adequate explants for a fast and better regeneration, presenting higher survival percentages than cotyledons (Montalban et al. 2011). Also several assays have led to the conclusion that organogenic potential of cotyledons decreased with explant age (López et al. 1996; Calixto & Pais 1997; Alonso et al. 2006; Alvarez et al. 2009).

Adventitious shoots induction and elongation

Culture medium formulation to induce direct organogenesis in *Pinus* spp. does not differ much from what has been described for the induction of axillary shoots. Like for axillary shoots propagation the most used cytokinin to induce adventitious shoots is BAP (see Table 1), being the adventitious bud formation dependent on the exposure time and concentration of cytokinin in the medium (Alonso et al. 2006). Although Montalbán and co-authors had tested other cytokinin in *Pinus radiata*, mT and zeatin (Z), concluded that BAP is the cytokinin that most favors organogenesis (Montalban et al. 2011).

While direct organogenesis procedures are very similar to the axillary shoots proliferation, indirect organogenesis shows higher similarity to somatic embryogenesis. Tang et al. (2005) presented an indirect organogenesis protocol for *Pinus elliottii*, selecting for callus induction phase, 2,4-dichlorophenoxyacetic acid (2,4-D), NAA and 2-isopentenyladenine (2iP), closer formulation from embryogenic callus induction phase (described in the next section). After the callus induction phase, shoots differentiation is obtained using IBA, BAP and TDZ.

Both direct and indirect organogenesis could benefit from the absence of light during the induction phase (Tang et al. 2006; Alvarez et al. 2009). Álvarez et al. (2009) concluded that bud induction in darkness prevents the cotyledons from becoming red and increases their bud and shoot-forming capacities.

The elongation of shoots is usually promoted in a culture medium with the same basic medium used for induction, but without growth regulators, and frequently with the addition of activated charcoal (Mathur & Nadgauda 1999; Alonso et al. 2006; Alvarez et al. 2009; Montalban et al. 2011).

Methods for rooting and acclimatization in organogenesis are common to those applied in axillary shoots proliferation protocols.

Somatic embryogenesis

Somatic embryogenesis (SE) is the process by which somatic cells or tissues differentiate into somatic embryos through a series of stages, different of the sexual process, the typical zygotic embryo development. Morphologically resemble zygotic embryos, the somatic embryos are bipolar and bear typical embryonic organs, the radicle, hypocotyl and cotyledons, but they develop via a different pathway (Barros 1999; von Arnold et al. 2002).

The first observation of somatic embryo formation was made at 1958 in *Daucus carota* cell suspensions (Steward et al. 1958; Reinert 1958). Since then, the potential for SE has been shown in a wide range of plant species. It is believed that SE can probably be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed (von Arnold et al. 2002).

Somatic embryogenesis is a wide applicability technique for studies relating to the physiology, genetics and biochemistry of embryo development and a is effective vehicle for genetic engineering (Guerra et al. 1999; Lipavská & Konrádová 2004). However the most promising application of SE is in high-value clonal forestry (Park et al. 1998). Through the implementation of industrial multi-varietal forestry (MVF; the use of tested high-value tree varieties in plantations), SE offers a new paradigm in tree breeding and deployment that is more flexible than the current seed orchard system and allows the capture of greater genetic gain (Park 2014). Also in most cases the somatic embryos or the embryogenic cultures can be cryopreserved, which is the other key technology for implementing MVF (Park & Bonga 2011).

SE can be expressed by two basic patterns: direct and indirect embryogenesis. In the first one it is suggested that pre-embryonic determined cells are present and require favorable inductive conditions to initiate embryo development. Indirect embryogenesis requires redetermination of differentiated cells and the acquisition of the embryogenic state prior to the initiation of embryo development (Yeung 1995). In *Pinus* spp. the processes described are usually of indirect SE (Table 2).

Table 2 - Summary of micropropagation induction by somatic embryogenesis for *Pinus* species. EM – embryogenic mass; PP – photoperiod; nd – not defined; *Se* – somatic embryos; ZE – zygotic embryos.

Species	Explant type	Basal Medium	Culture conditions	Carbon source	PGR	Response	Acclimated plantlets	Reference
P. armandii	Immature ZE	½ EM	25ºC, dark	1% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Maruyama et al. 2007
P. banksiana	Immature ZE	mLV	24ºC, dark	1% sucrose	CPPU	EM	nd	Park et al. 2006
P. brutia	Immature ZE	DCR	24ºC, dark	nd	2,4-D and BAP	EM and *Se*	No	Yildirim et al. 2006
P. bungeana	Immature ZE	DCR	23ºC, dark	3% sucrose	2,4-D and BAP	EM and *Se*	No	Zhang et al. 2007
P. caribaea	Immature ZE	LPG	23ºC, dark	3% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	David et al. 1995
P. elliottii	Immature ZE	EVX: modified DCR	23ºC, dark	3% sucrose	NAA and BAP	Extrusion callus	No	Liao & Amerson 1995
	Immature ZE	DCR	20-25ºC, dark	2% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Newton et al. 1995
	Immature ZE	1369B (described on the article) + paclobutrazol	23-25ºC, dark	1.5% maltose	2,4-D and BAP	EM	No	Pullman et al. 2005
P. halepensis	Immature ZE	DCR + ED aminoacids	21ºC, dark	3% sucrose	Kinetin and 2,4-D or BAP, 2,4-D and NAA	EM, *Se* and emblings	Yes	Montalbán et al. 2013
P. heldreichii	Immature ZE	GD	25ºC, dark	3% sucrose	2,4-D and BAP	EM	No	Stojičić et al. 2007
P. kesiya	Immature ZE	DCR	23ºC, dark	3% maltose	2,4-D, BAP and NAA	EM, *Se* and emblings	nd	Choudhury et al. 2008
P. nigra	Immature ZE	DCR	23ºC, dark	2% maltose	NAA or 2,4-D	EM, *Se* and emblings	nd	Salajova & Salaj 2005
P. oocarpa	Immature ZE	1250 (Pullman et al., 2006)	Dark	3% sucrose	2,4-D, ABA and BAP	EM, *Se* and emblings	Yes	Lara-Chavez et al. 2011
P. patula	Immature ZE	DCR	25ºC, dark	3% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Jones & Van Staden 1995
	Apical shoot of mature tress	DCR	4ºC, dark + 25ºC, dark	3% maltose	No PGRs + BAP, NAA and 2,4-D	EM, *Se* and emblings	nd	Malabadi & Van Staden 2005

Species	*Explant type*	*Basal Medium*	*Culture conditions*	*Carbon source*	*PGR*	*Response*	*Acclimated plantlets*	*Reference*
P. pinaster	Immature ZE	DCR	23ºC, dark	2% sucrose	2,4-D and BAP	EM and Stage 2 and 3 *Se*	nd	Miguel et al. 2004
	Immature ZE	mLV	25ºC, dark	3% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Lelu-Walter et al. 2006
	Immature ZE	mLV	24ºC, dark	1% sucrose	CPPU	EM	nd	Park et al. 2006
	Immature ZE	mLV	26ºC, dark	2% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Humánez et al. 2012
	Immature ZE	WV5	23ºC, dark	3% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Alvarez et al. 2013
P. pinea	Immature ZE	mLV_2	23ºC, dark	1% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Carneros et al. 2009
P. radiata	Immature ZE	EDM6	24ºC, dark	3% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Walter et al. 2005
	Immature ZE	mLV	24ºC, low light	3% sucrose	2,4-D and BAP	EM	nd	Hargreaves et al. 2009
	Immature ZE	EDM	21ºC, dark	3% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Montalbán et al. 2010
P. rigida x P. taeda	Immature ZE	P6	24ºC, dark	3% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Kim & Moon 2007
P. roxburghii	Apical shoot of mature tress	DCR	4ºC, dark + 25ºC, dark	3% maltose	No PGRs + BAP, NAA and 2,4-D	EM, *Se* and emblings	Yes	Malabadi & Nataraja 2006
P. strobus	Immature ZE	mLV	24ºC, dark	2% sucrose	2,4-D and BAP	EM, *Se* and emblings	Yes	Klimaszewska et al. 2001
	Immature ZE	mLV	24ºC, dark	1% sucrose	2,4-D and BAP	EM	nd	Park et al. 2006

Species	*Explant type*	*Basal Medium*	*Culture conditions*	*Carbon source*	*PGR*	*Response*	*Acclimated plantlets*	*Reference*
P. sylvestris	Immature ZE	mLV	24ºC, dark	3% sucrose	2,4-D and BAP	EM	nd	Park et al. 2006
P. taeda	Mature ZE	LOB	23ºC, dark	3% sucrose	2,4-D, BAP and Kinetin	EM, *Se* and emblings	Yes	Tang et al. 2001
	Immature ZE and *Se*	505, described by the authors	23-25ºC, dark	1.5% maltose	NAA and BAP	Extrusion callus	No	Pullman & Johnson 2002
	Immature ZE	1253 (described on the article) + paclobutrazol	23-25ºC, dark	1.5% maltose	2,4-D and BAP	EM, *Se* and emblings	nd	Pullman et al. 2005

Pinus SE proceeds through four steps: 1. Initiation of embryogenic cultures; 2. Proliferation of embryogenic cultures; 3. Maturation of somatic embryos; 4. Germination/Conversion into plants (Newton et al. 1995; Pullman & Bucalo 2011).

Explants type and in vitro establishment

The SE techniques developed and used for coniferous species start generally from young explants, and in the case of *Pinus* spp. research carried out over the last two decades has proven that SE is initiated most efficiently from immature zygotic embryos (Klimaszewska et al. 2007). However initiation and establishment of embryogenic cultures from vegetative shoot apices of mature trees or from mature zygotic embryos of *Pinus* spp. has been achieved only in a few species, with a very low maturation rate (Tang et al. 2001; Malabadi & Van Staden 2005; Malabadi & Nataraja 2006; Park & Bonga 2011).

For several conifers, mature zygotic embryos are already non-responsive and immature embryos have to be used, because the initiation rate gradually diminishes as the zygotic embryo matures (Bonga et al. 2010).

The zygotic embryo in pine seeds cleaves at the 16-cell stage, forming four new embryos each composed of four cells, process known as polyembryony (Klimaszewska et al. 2007, Bonga et al. 2010). Bonga and co-authors (2010) speculate that in cultures of these species, the cleavage process repeats itself each time the newly formed embryos reach the 16-cell stage when exposed to auxin *in vitro*, thus forming a large number of somatic embryos. This early stage of seed development corresponds to 3-6 weeks post-fertilization, when the dominant zygotic embryo is at a pre-cotyledonary stage of development (Becwar et al. 1990). Regarding shoot apices induction, these tissues contain small areas that are more morphogenetically competent than surrounding ones, Bonga et al. (2010) claim that the expression of this totipotency is inhibited by surrounding tissues that have to be removed from the explant before a morphogenetic response can be expected.

Besides the type of explant, genetic background has significant effect on initiation of somatic embryogenesis (Aronen & Pehkonen 2009). That's the reason why several genotypes of the same species are evaluated during the development of SE protocols, to find and select those that are responsive for further experiments (Liao & Amerson 1995;

Pullman & Johnson 2002; Miguel et al. 2004; Carneros et al. 2009; Hargreaves et al. 2009; Montalbán et al. 2013).

Cold storage of cones prior to seed preparation for explanting can significantly increase initiation results (Pullman & Bucalo 2011). After surface-sterilization of the seeds the megagametophytes are excised, and in most cases used as explant by placing on medium to permit the extrusion of embryogenic tissue from the micropylar end (e.g. Newton et al. 1995; Park et al. 2006; Montalbán et al. 2010; Alvarez et al. 2013). It has been suggested that the relative success achieved using this method is due to the fact that as the megagametophyte is attached to the embryo there is a reduction in excision stress. The gametophyte provides the embryo with some of its natural nutrients and phytohormones, thus making culture easier (Pascoe 2002). In other cases the immature zygotic embryos isolated from the megagametophyte is used as explant with higher success in the induction of embryogenic masses (Lelu-Walter et al. 2006).

Initiation and Proliferation of embryogenic cultures

The initiation of the embryogenic tissue within the SE pathway is a vital step. The embryogenic tissue has an white to translucent and mucilaginous appearance. Microscopic examination shows that the embryogenic cultures consist of a mixture of early stage somatic embryos containing an embryonal mass (globular clumps of densely cytoplasmic cells) with attached suspensor-like cells (Pascoe 2002).

Somatic cells within the plant contain all the genetic information necessary to create a complete and functional plant. The induction of somatic embryogenesis must then consist of the termination of a current gene expression pattern in the explant tissue, and its replacement with an embryogenic gene expression program (von Arnold et al. 2002). One possible mechanism for downregulation of current gene expression is DNA methylation, which is influenced by auxins (LoSchiavo et al. 1989). In fact, in most designs embryogenesis induced *in vitro*, auxins and among them 2,4-D are considered the substances responsible for the onset of dedifferentiation process, changing determination and giving new competences to the responsive cells present in the explant (Guerra et al. 1999). Therefore 2,4-D is the auxin most used on *Pinus* spp. SE initiation, in concentrations close than 9 μM for immature embryos (e.g. Newton et al. 1995;

Klimaszewska et al. 2001; Stojičić et al. 2007; Alvarez et al. 2013), and with higher concentrations to mature zygotic embryos (Tang et al. 2001). The auxin NAA is also used with some regularity in SE initiation in *Pinus* spp. (Liao & Amerson 1995; Pullman & Johnson 2002; Malabadi & Van Staden 2005; Malabadi & Nataraja 2006), abscisic acid (ABA) is occasionally used at this stage although in combination with 2,4-D (Lara-Chavez et al. 2011) and is practically transversal between the protocols established for *Pinus* spp. the use of BAP as cytokinin (see Table 2).

Several formulations of media are used in pine SE: DCR, or modified DCR (Gupta & Durzan 1985); EDM (Walter et al. 1998); modifications of P6-based medium (Teasdale et al. 1986) as described by Pullman and Johnson (2002); WV5 (Coke 1996); mLV (Litvay et al. 1985) . Most of these culture media are used also for the other micropropagation techniques, but for SE the media are typically enriched with sources of organic nitrogen (L-glutamine and casein hydrolysate or with a mixture of several amino acids), because they favour the production of a greater number of somatic embryos formed of better quality (Higashi et al.1996; Klimaszewska et al. 2007).

Carbon is provided in most cases by the addition of sucrose (1-3%) like for the other micropropagation pathways, but other saccharides were tested as well (e.g. glucose, fructose, maltose, lactose, cellobiose, mannitol, sorbitol, myo-inositol) in *Pinus* spp. and proved to be less efficient in most cases (e.g. Lara-Chavez et al. 2011), although in some species the best results were achieved when some of these alternative carbohydrates were used as the medium carbon and energy source (Lipavská & Konrádová 2004). For example, Pullman et al. (2005) used 1.5% maltose in the culture medium during the EM proliferation of *Pinus taeda.*

Initiation step of SE is not perfectly synchronized and may occur between 2 and 16 weeks, however, most explants produce macroscopically identifiable embryogenic mass between 6-10 weeks (Klimaszewska et al. 2007).

After initiation, embryogenic mass (EM) can be maintained and proliferated in the same culture medium used for initiation, or in a medium similar that usually contain reduced hormones. EM can be kept on semi-solid medium, like in initiation, however, for large-scale propagation it is usually better to establish suspension cultures. Liquid media have the advantage of faster growth rates, decreasing variation, simplifying preparation of cells

for cryostorage, ease of visualizing somatic embryos, and automation of cell suspension transfer (Pullman & Bucalo 2011).

Although embryogenic cultures of some species and some genotypes can be subcultured for a prolonged period on medium containing PGRs, without lose their full embryogenic potential, in most cultures the embryogenic potential decreases with prolonged culture and is eventually lost. Furthermore the occurrence of somaclonal variation increases with prolonged culture (von Arnold et al. 2002) .

For many species, for example *Pinus pinaster* (Klimaszewska et al. 2009), the cultures will remain embryogenic only through a limited number of subcultures and can be maintained in an embryogenic state only by cryopreservation shortly after establishment of the culture or by inducing secondary embryogenesis from mature somatic embryos (Bonga et al. 2010).

Maturation of somatic embryos

During the maturation stage, the somatic embryos undergo various morphological and biochemical changes, they accumulate storage products that exhibit the same characteristics as those of the zygotic embryos. The synthesis and deposition of storage during somatic and zygotic embryogenesis are usually regulated through ABA- and water-stress-induced gene expression (Dodeman et al. 1997).

ABA has been recognised as an important hormone, with a role in early to late maturation, which probably is due to its activity on the normal development of the seeds, where ABA accumulates during mid to late stages of development preventing the embryos from germinating precociously (Attree & Fowke 1993; Misra 1994). So an inorganic and organic medium composition used for this stage of SE is usually the same as for previous stages, with the replacement of both auxin and cytokinin by ABA (Klimaszewska et al. 2007). For *Pinus* spp. there are successful maturation protocols with concentrations of ABA between 10 and 150 μM (see Table 3).

Another critical factor that was discovered to promote development of large numbers of somatic embryos was the restriction of water availability (Klimaszewska & Smith 1997). This fact is related to the natural maturation of seed embryos that is generally concluded

with some degree of drying, which results in a gradual reduction of metabolism as water is lost from the seed tissue and the embryo passes into a metabolically inactive, or quiescent, state (von Arnold et al. 2002). This restriction of water availability can be achieved by physical or osmotic means, or by the combination of both.

Different osmotic agents, including low (e.g. sugars) and high (e.g. polyethylene glycols-PEG) molecular mass compounds can provide low osmotic potential medium (von Arnold et al. 2002). As is can be observed on Table 3, several of the SE protocols described for *Pinus* spp. increase the sugar concentration on this phase. In alternative there are also other protocols where PEG was added to the medium, or that use a combination of the two methodologies (Table 3).

The other method to reduce the water availability to the cultured cells is by physical means, which can be accomplished by increasing the medium gel strength, i.e. the gelling agent concentration in the culture medium (Klimaszewska et al. 2007). Several researchers choose to use both techniques, the use of osmotic and physical means to reduce the water availability (e.g. Klimaszewska et al. 2001; Miguel et al. 2004; Carneros et al. 2009; Montalbán et al. 2013).

A step prior to maturation with activated charcoal (AC) can also improve further maturation, per example for *Pinus elliottii* better results were achieved with the use of AC, suggesting that excess of both auxins and cytokinins may be inhibitory to maturation (Newton et al. 1995). For *Pinus pinaster* there is also reports that indicate the use of AC at this stage as positive for maturation (Miguel et al. 2004; Humánez et al. 2012).

Table 3 - Summary of SE maturation for *Pinus* species. gFM – grams of fresh mass; nd – not defined; *Se* – somatic embryos.

Species	***PGR***	***Carbon source***	***Gelling agent***	***PEG***	***Response***	***Reference***
P. armandii	100 mM ABA	5% maltose	0.3% gellan gum	10% PEG_{4000}	Max. 101 *Se* per 500 mg FM	Maruyama et al. 2007
P. brutia	80 mM ABA	5% sucrose	1% gellan gum	3.75% PEG_{4000}	Max. 50% lines with maturation ability	Yildirim et al. 2006
P. bungeana	0.5 mM IBA	5% sucrose	nd	---	Max. 30% lines with maturation ability	Zhang et al. 2007
P. caribaea	15 mM ABA	2% sucrose	0.6% agarose	---	nd	David et al. 1995
P. elliottii	10 mM ABA	3% sucrose	1% gelrite	---	215 stage 3 *Se* and 65 stage 4.	Newton et al. 1995
P. halapensis	75 mM ABA	6% sucrose	0.9% gelrite	---	7 to 716 *Se* / gFW	Montalbán et al. 2013
P. kesiya	35 mM ABA	6% maltose	0.5% gellan gum	---	35 to 51 *Se* / gFW	Choudhury et al. 2008
P. nigra	95 mM ABA	9% maltose	0.4% phytagel	---	59 to 135 *Se* / gFW	Salajova & Salaj 2005
P. ocarpa	40 mM ABA	6% maltose	0.6% phytagel	12% PEG_{8000}	20 *Se* / mgFW	Lara-Chavez et al. 2011
P. patula	38 mM ABA	6% maltose	0.3% gelrite	10% PEG_{4000}	51.4 *Se* / gFW	Jones & van Staden 2001
P. pinaster	120 mM ABA	2% sucrose	1% phytagel	10% PEG_{4000}	29% lines with maturation ability	Miguel et al. 2004
	80 or 120 mM ABA	6.8% sucrose	1% gellan gum	---	24 to 441 *Se* / gFW	Lelu-Walter et al. 2006
	80 mM ABA	6.8% sucrose	1% gelrite	---	Max. 274 stage 3 *Se* / gFW	Humánez et al. 2012
	80 mM ABA	6% sucrose	0.9% gelrite	---	100 to 300 embryos / gFW	Alvarez et al. 2013
P. pinea	121 mM ABA	6% sucrose	1% gelrite	---	Max. 3 *Se* per 250 mg fresh embryogenic tissue	Carneros et al. 2009
P. radiata	15 mM ABA	3% sucrose	0.6% gelrite	---	nd	Walter et al. 2005
	60 mM ABA	6% sucrose	0.9% gelrite	---	600 *Se* / gFW	Montalbán et al. 2010
P. rigida x P. taeda	100 mM ABA	6.8% maltose	1.2% gellan gum	---	Max. 224 *Se* / gFW	Kim & Moon 2007
P. strobus	120 mM ABA	6% sucrose	1% phytagel	---	122,6 *Se* / gFW	Klimaszewska et al. 2001
P. taeda	151.3 mM ABA	1.5% sucrose	0.7% agar	7.5% PEG_{6000}	5 to 8 *Se* per ml fresh culture	Tang et al. 2001
	19 mM ABA	2% maltose	0.25% gelrite	13% PEG_{8000}	Max. 225 *Se* per ml fresh culture	Pullman et al. 2003
	5.2 mM ABA	2% maltose	0.25% gelrite	13% PEG_{8000}	50% lines with maturation ability	Pullman et al. 2005

Germination/Conversion into plants

For an efficient germination the quality of the somatic embryos obtained is very important, because only mature embryos with a normal morphology and which have accumulated enough storage materials and acquired desiccation tolerance at the maturation end, could develop into normal plants, comparable to seedlings (von Arnold et al. 2002).

Somatic embryos germination is carried out most frequently on a semi-solid medium without PGR, being common the reduction of nutrients at this stage (e.g. Lara-Chavez et al. 2011; Pullman & Bucalo 2011; Montalbán et al. 2013). It is also frequent the presence of AC on the germination medium to adsorve residual PGR (Pullman & Bucalo 2011).

Some treatments increase the germination of pine somatic embryos such as darkness or the light reduction on the first 7-14 days of culture (e.g. Klimaszewska et al. 2001; Lelu-Walter et al. 2006; Lara-Chavez et al. 2011; Humánez et al. 2012). This ensures elongation of hypocotyls, reduces anthocyanin synthesis and facilitates later handling. It has been also suggested that a treatment of partial drying of the embryos following ABA maturation improves germination, because it seems to reduce the level of endogenous ABA (Li et al. 1997). Lara-Chavez et al. (2011) described the treatment of high-humidity-desiccation of *P. oocarpa* mature somatic embryos in order to accelerate the root development. The same treatment was used before to *P. radiata* and leads also to improvements on germination (Pascoe 2002).

Once the somatic embryos convert to plantlets, after 12 to 14 weeks depending on the species, the process of acclimatization is made like it was described before to plantlets obtained from axillary shoot proliferation.

Although for some species of *Pinus* plant conversion rates higher than 75% have been achieved in some ECL (e.g. Klimaszewska et al. 2001; Montalbán et al. 2010; Lara-Chavez et al. 2011; Humánez et al. 2012; Alvarez et al. 2013), unfortunately, the germination of *Pinus* embryos depends of the genotype and is often difficult to achieve (Pullman & Bucalo 2011).

Pinus spp. *germplasm preservation*

The conservation of plant genetic resources is considered strategic for a country due to their social, economic and environmental values as well as their use in classical and modern breeding programs (Panis & Lambardi 2005; Aguiar et al. 2011).

One of the most important reasons for the plant genetic resources preservation is related to the risk of its extinction. Lynch and co-authors (2007) pointed that it has been defended that climate change presents real risks, and that these risks are of such magnitude, that will be felt on a global scale. They referred also that if warming continues, more than a million species could be extinct by 2050.

The two most common approaches to plant germplasm conservation are the *in situ* and *ex situ* conservation. It is called *in situ* to the conservation of native species populations kept in their natural environment. *Ex situ* conservation of germplasm collections is used to maintain genetic variability of the species outside their natural habitat, which includes the storage of seeds, pollen and DNA, *in vitro* and field genebanks and botanical gardens. The *in situ* approach encompasses genetic reserves, on-farm and home garden conservation. It has been emphasized that for any given genepool, a number of different complementary approaches and methods are necessary for a safe, efficient and cost-effective conservation (Engelmann 2000; Netto 2010). Although field collections offer the most practical approach to conservation, as such plant genetic resources can be accessed and observed easily, other *ex situ* conservation techniques are essential for the continued and sustainable use and exploitation of these biological assets. Since plant genetic resources maintained as field collections will be particularly vulnerable to the effects of climate change, as weather patterns become more erratic and extreme, and to the action of pests and diseases (Panis & Lambardi 2005; Lynch et al. 2007).

Most *Pinus* germplasm collections have been conserved in field, but because of its longevity cycle and the need for large plantation areas, the permanence of this resource in the field will depend on the economic value of genetic material. So the most conserved *Pinus* germplasms are those that have been involved in breeding programs and are well established on seed collection areas and seed orchards (Aguiar et al. 2011).

For genotypes that are propagated vegetatively the conservation emphasis has been on *ex situ* techniques including field genebank and *in vitro* storage, which should be considered as complementary strategies, since field collections offer many important advantages to plant breeders and germplasm conservationists (Engelmann 2000). *In vitro* conservation, which can be achieved by micropropagation of shoots or SE, offers the advantage of storage under secure, controlled environmental conditions and supports the distribution of disease-free germplasm. However, *in vitro* approaches do have some significant drawbacks, there is always the risk of losing material due to microbial contamination, human error, equipment failure, and the effects of time-related epigenetic and genotypic change, also known as somaclonal variation, which frequency increases with repeated subculturing. Although medium-term storage, under reduced (slow) growth, is a cost-effective option used by many international genebanks to conserve vegetatively propagated plant species, it is not suitable for long-term storage due to the risks of selection, stress and epigenetic change (Panis & Lambardi 2005; Miguel & Marum 2011). As already mentioned, for many *Pinus* spp. SE cultures remain the embryogenic capacity only through a limited number of subcultures and need to be cryopreservated in order to be maintained in an embryogenic state (Marum et al 2004; Bonga et al. 2010). The cryostorage of tissues may prevent any unfavorable changes that take place during successive passages *in vitro*. This method makes it possible to limit the number of passages and the risk of microbial contamination and somaclonal variation in the stored cultures (Hazubska-Przybył et al. 2010).

Taking into account the existing risks of losing genetic material with the *in situ* and *ex situ* conservation methods described before, the alternative strategies to ensure more efficient and economic conservation and reduction of germplasm loss have to be developed. Cryopreservation is the only realistic long-term storage option, for vegetatively propagated plant genetic resources that cannot be conserved by seed banking (Sudarmonowati et al. 2000).

Cryopreservation

Plant material can be stabilized at long-term by subjecting them to cryogenic temperatures (LN, -196°C). Stabilizing cells at cryogenic temperatures is called cryopreservation, an applied aspect of cryobiology, or the study of life at utra-low temperatures (Simione 1998).

The main advantage of cryopreservation is that once material has been successfully cooled to LN temperatures, it can be conserved in principle indefinitely, because at these ultra-low temperatures no metabolic processes occur (Engelmann 2004). Other advantages are the low costs of storage, minimal space requirements and reduced labour maintenance compared to living collections and even when compared to maintenance of tissue cultures at room temperature (Kaczmarczyk et al. 2012).

Advances in cryopreservation technology have led to methods that allow ultra-low temperature maintenance of many different types of plant material, such as seeds, embryonic axes isolated from seeds, vegetative propagated plant material, including apical or axillary buds, pollen, somatic embryos, and embryogenic cultures (Hazubska-Przybył et al. 2010). Despite of the advances made, cryopreservation of non cold-hardy plants, especially tropical species, which are not intrinsically tolerant to low temperature and desiccation, has been less extensively investigated (Takagi 2000).

Damage to plant cells due to freezing

Plant tissues and suspensions are constituted by water-rich cells that will not survive exposure to the ultra-low temperature of liquid nitrogen (Reinhoud et al. 2000). Water is one of the most important components of living systems, conferring structural order and regulating every life process. The control of water status during cryopreservation is the key factor in developing successful cryoprotective strategies and limiting cryo-injury, since 95% of the water present in biological tissues is “free” and will convert to ice during freezing, causing irreversible damage. The dehydration of the cells during the cryopreservation procedure, can strongly reduced the chance of intracellular crystals formation. But this procedure has to be optimized because dehydration can also leads to damage of the cells. At a slow cooling rate and in the event where crystallization is first induced in the extracellular compartment, damage is attributed to the extreme osmotic dehydration which occurs when intracellular, unfrozen water moves from inside to outside the cell to compensate for the water vapour deficit as water freezes in the extracellular component (Dumet & Benson 2000).

Exposure to low temperatures can also result in inactivation of proteins that are sensitive to cold, leads to damage on the cells membrane. Moreover, the stress originated by freezing

may result in the formation of free radicals, which can cause lipid peroxidation, proteins denaturation and DNA mutations (Cadenas 1989; Benson et al. 1992).

Cryoprotective agents and pre-treatments

The use of cryoprotectants helps decreasing cryo-injury specifically intracellular ice formation and dehydration (Lynch et al. 2007). Cryoprotectants have several functions during the freezing process, like freezing point depression that permits to encourage a greater dehydration of the cells prior to intracellular freezing, promoting delay on intracellular freezing and minimizing the solution effects when they penetrate the cell. The choice of a cryoprotective agent is dependent upon the type of cell to be preserved. For most cells, glycerol is the agent of choice because it is usually less toxic than dimethyl sulfoxide (DMSO). However, DMSO is more penetrating and is usually the agent of choice for larger, more complex cells (Simione 1998). Osmotic dehydration can be obtained through the application of non-penetrating cryoprotective substances, such as sugars, sugar alcohols and high molecular weight additives like polyethylene glycol (PEG) (Panis & Lambardi 2005). For plant cells usually the combinations of cryoprotectans are more effective than agents used singly. It has been reported that the regrowth percentage of germplasm after cryopreservation is higher when mixing cryoprotectans (Kami 2012).

The capacity of plant tissues or cells suspensions to adapt to environmental stress, caused by low temperatures, can be employed in a pre culture period prior to the cryopreservation procedure (Dumet & Benson 2000). Being the most common the use of sugar-rich media, 0,2 to 0,7M of sucrose, maltose or sorbitol between others. In fact the selection of the sugar used on pre-culture is common on cryopreservation protocols optimization. Marum et al. (2004) tested the effect of sorbitol, maltose, glucose and sucrose on *Pinus pinaster* embryogenic cultures cryopreservation, getting better results when maltose was used on pretreatment. On the other hand Salaj et al. (2011) for *Pinus nigra* embryogenic cultures didn't find major differences between the use of maltose or sucrose. The use of sugar-rich media for pre culture, semi-solid or liquid, was also described for *P. sylvestris* (Häggman et al. 1998; Latutrie & Aronen 2013), *P. radiata* (Hargreaves et al. 2002), *P. roxburghii* (Mathur et al. 2003) and *P. kesiya* (Kalita et al. 2012).

Two major hypotheses exist concerning the mode of action of sugar in desiccation tolerance. First, it could replace the water molecules involved in the maintenance of macromolecular structure; secondly, it could induce vitrification of the intracellular medium at biological temperatures (Crowe et al. 1988; Williams & Leopold 1989).

Exposure to non-lethal temperature stress prior to the cryopreservation procedure also can enhance survival rates (Reinhoud et al. 2000). This procedure was described for example for *P. sylvestris*, where the embryogenic cultures where maintained at 5ºC for 14 days prior to cryopreservation (Häggman et al. 1998).

In the last three decades a number of different plant cryopreservation protocols have been developed, which can be divided in two basic strategies: classical slow-cooling or vitrification-based techniques. The choice of cryopreservation method to achieve the highest survival rates is largely dependent on the plant species and tissue type that is being cryostored (Takagi 2000; Engelmann 2000; Kaczmarczyk et al. 2012).

Classical slow cooling cryopreservation

The classical method for cryopreservation of cultured plant cells is the two-step or slow-cooling method developed in the late 1970s (Withers & King 1980). These techniques involve slow cooling down to a defined pre-freezing temperature, usually at, or around, -40ºC, the temperature of homogeneous ice formation, followed by rapid immersion in liquid nitrogen (Engelmann 2000; Benson et al. 2005).

In slow cooling cryogenic systems, cells are dehydrated as a result of a non-equilibrium vapour pressure gradient being formed at the point of extracellular ice nucleation. This precipitates the movement of unfrozen water from the intracellular compartment, reducing the amount of freezable water (Benson et al. 2005). In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation upon subsequent immersion of the plant cells in liquid nitrogen. However, too intense freeze induced dehydration can incur different damaging events owing to concentration of intracellular salts and changes in the cell membrane (Meryman et al. 1977).

In these techniques, cells are cryoprotected using colligative, penetrating cryoprotectants, which lower the freezing point and ameliorate the damaging effects of excessive solute concentration (Benson et al. 2005). Carefully regulated slow cooling can be achieved using an controlled-rate freezing equipment or a laboratory freezer like the static solvent containing called "Mr Frosty" may be employed (Harding et al. 2004). This last one providing a cooling rate of 0.99°C/min (Cyr 2000). The main limitation of this procedure on a large scale is the need of an expensive controlled-rate freezing equipment (Reinhoud et al. 2000).

Classical slow cooling cryopreservation techniques have been successfully applied to undifferentiated culture systems such as cell suspensions and callus, and although these techniques could be employed also for freezing apices of cold-tolerant species, their utilization with high survival for tropical species was exceptional (Reed & Chang 1997; Takagi 2000).

Efforts in conifer SE cryopreservation have focused primarily on cultures that are comprised of early stage *Se*, commonly referred to as embryogenic masses (EM) (Cyr 2000). In fact there are several crypreservation protocols based on slow cooling techniques described for *Pinus* spp. EM, such as *P. sylvestris* (Häggman et al. 1998; Latutrie & Aronen 2013), *P. radiata* (Hargreaves et al. 2002), *P. roxburghii* (Mathur et al. 2003), *P. pinaster* (Marum et al. 2004; Álvarez et al. 2012) or *P. nigra* (Salaj et al. 2011).

Vitrification-based techniques

In 1985, a new method for cryopreservation of animal cells was reported, where the cells were preserved in liquid nitrogen in the absence of ice (Rall & Fahy 1985). This method was known as vitrification. The first successful vitrification procedures for plant cell suspensions were developed in 1989 for rapeseed and asparagus (Langis et al. 1989; Uragami et al. 1989). Currently, this is considered one of the most important approaches to plant cryoprotection (Dumet & Benson 2000).

Vitrification describes the phase-transition of water from the liquid to an amorphous glassy state, which lacks crystalline structure. So the vitrified state is characterized by the glass transition temperature, which is the temperature at which a liquid forms a glass (Zámečník et al. 2012). This condition occurs in biological systems when the solute concentration and

viscosity becomes sufficiently high to inhibit ice nucleation (Lynch et al. 2007). This high viscosity state can be achieved by evaporative desiccation (e.g. in sterile air streams, or over silica gel) and osmotic dehydration and/or the application of penetrating cryoprotectants (Benson et al. 2005). As a consequence, cryo-injury imposed by intra and extracellular freezing and the associated solution effect is avoided (Dumet & Benson 2000).

Vitrification-based techniques offer other practical advantages compared with classical slow-cooling protocols. They are more appropriate for freezing complex organs, allowing the use of samples with relatively large size (shoot-tips of 0.5 to 2–3 mm and somatic embryos), which contain a variety of cell types. They are also ultra-rapid freezing techniques and by precluding ice formation in the system, the vitrification-based procedures simplify the cryogenic procedure and eliminate concerns for the potentially damaging effects of intra- and extracellular crystallization (Uragami et al. 1989; Engelmann 2000).

A common step to all previously described cryopreservation methods is thawing and regeneration of the plant material. Surviving cells or tissues after cryopreservation readily succumb due to different environmental agents because they have been injured by the dehydration or temperature change during the cryopreservation procedure. Moreover, when plant specimens were injured by the cryopreservation process, polyphenol can be produced (Kami 2012).

During rewarming the ice nucleation may be formed when samples are slowly rewarmed above the glass transition temperature and homogeneous ice nucleation point (≥ −40 °C) (Martinez-Montero & Harding 2015). To prevent further injury for thawing a rapid warming in an water bath is required, avowing recrystallization and ensuring a proper recovery of the cryopreservated material. Thawing temperatures ranging from 20°C to 40°C have been proposed for woody species (Panis & Lambardi 2005). Due to the polyphenol presence, it has been reported that regeneration of tissues after preservation can increased when activated charcoal is mixed with a culture medium (e.g. Hargreaves et al. 2002).

Research objectives

As previously mentioned this project was conducted in collaboration with the company KLÓN, Innovative Technologies from Cloning. The development of strategies for cloning and preservation of species/genotypes with high agroforestry value, including the species and hybrid under study in this work, were pointed out as crucial to the species and hybrid breeding program.

In this context, the main goal of the present work was to establish efficient protocols for *in vitro* plant regeneration, by micropropagation techniques, and preservation of the produced germplasm bank. The specific objectives of this work were the following:

- To develop an efficient protocol for rapid multiplication of *Pinus elliottii* var. *elliottii* by axillary shoot proliferation;
- Assessment to the physiological performance and the ploidy stability of *Pinus elliottii var. elliottii* micropropagated plants when compared to seed-derived plants;
- Establishment of a successful protocol of SE for *P. elliottii x P. caribaea*, by testing different factors: the influence of the genotype and culture media formulations on the initiation rates and maturation capacity of the ECLs initiated;
- True-to-typeness analysis along the different phases of the SE process by DNA-ploidy determination of the embryogenic masses and emblings;
- Development of a cryopreservation protocol for the interspecific hybrid EM, testing the effect of several cryopreservation parameters on the recovery ECLs;
- Evaluation of the embryogenic potential, morphology and genetic stability of the ECLs after cryopreservation.

References

Aguiar, A.V., I. Wendling, and J. Y. Shimizu. 2013. "Cultivo de Pínus: Propagação Vegetativa." *Revista Da Madeira* 134.

Aguiar, Ananda Virgínia de, Valderês Aparecida de Sousa, Elenice Fritzsons, and José Elidney Pinto Junior. 2011. "Programa de melhoramento de *pinus* da Embrapa Florestas." Embrapa Florestas.

Aitken, Jenny, Kathryn J. Horgan, and Trevor A. Thorpe. 1981. "Influence of Explant Selection on the Shoot-Forming Capacity of Juvenile Tissue of *Pinus radiata*." *Canadian Journal of Forest Research* 11 (1). NRC Research Press Ottawa, Canada: 112–17. doi:10.1139/x81-015.

Almeida, Natalie Ferreira de. 2011. "*Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis* wood quality evaluation for veneer production and plywood manufacturing." Universidade de São Paulo Escola Superior de Agricultura "Luiz de Queiroz."

Alonso, Pablo, Paloma Moncaleán, Belén Fernández, Ana Rodríguez, Maria Luz Centeno, and Ricardo J. Ordás. 2006. "An Improved Micropropagation Protocol for Stone Pine (*Pinus pinea* L.)." *Annals of Forest Science* 63 (8). EDP Sciences: 879–85. doi:10.1051/forest:2006071.

Alvarez, J. M., J. Majada, and R. J. Ordas. 2009. "An Improved Micropropagation Protocol for Maritime Pine (*Pinus pinaster* Ait.) Isolated Cotyledons." *Forestry* 82 (2): 175–84. doi:10.1093/forestry/cpn052.

Alvarez, José M, Natalia Bueno, Millán Cortizo, and Ricardo J Ordás. 2013. "Improving Plantlet Yield in *Pinus pinaster* Somatic Embryogenesis." *Scandinavian Journal of Forest Research* 28 (7): 613–20.

Álvarez, José M., Millán Cortizo, and Ricardo J. Ordás. 2012. "Cryopreservation of Somatic Embryogenic Cultures of *Pinus pinaster*: Effects on Regrowth and Embryo Maturation." *Cryoletters* 33 (6). Cryoletters: 476–84.

Anália, G.P. 2010. "Propagacion in Vitro de Um Híbrido Entre *Pinus elliottii* var. *elliottii Y Pinus caribaea* var. *hondurensis.*" Universidad Nacional Del Nordeste, Corrientes.

Andrade, SRM. 2002. *Princípios Da Cultura de Tecidos Vegetais*. 1Ed. ed. Vol. 58. Cerrados: Planaltina: Embrapa.

Andrejow, Gisela Maria Pedrassani. 2006. "Minijardim clonal de *Pinus taeda* L." Universidade Federal do Paraná.

Aronen, T, and T Pehkonen. 2009. "Somatic Embryogenesis of Scots Pine–advances in Pine Tissue Culture at Metla." In *Working Papers of the Finnish Forest Research Institute*, 114: 68–71.

Arya, R. S., and M. S. Haque. 1982. "Air Layering in *Pinus patula* Schi. And Cham. And *Pinus caribaea* Morelet. var. *hondurensis*." *Indian Forester* 108 (1): 66–68.

Assis, TF. 1997. "Propagação Vegetativa de Eucalyptus Por Microestaquia." In *Conferência IUFRO Sobre Silvicultura E Melhoramento de Eucaliptos*, 1:300–304. Salvador: Colombo: Embrapa, CNPF.

Assis, TF, and MDV Resende. 2011. "Genetic Improvement of Forest Tree Species." *Crop Breeding and Applied Biotechnology* S1: 44–49.

Attree, S. M., and L. C. Fowke. 1993. "Embryogeny of Gymnosperms: Advances in Synthetic Seed Technology of Conifers." *Plant Cell, Tissue and Organ Culture* 35 (1): 1–35.

doi:10.1007/BF00043936.

Azevedo, C, J Maia, C Lopes, and F Gomes. 2001. "Micropropagação Do Pinheiro Bravo (*Pinus pinaster* Aiton)." *Melhoramento* 37: 62–66.

Barros, Levi de Moura. 1999. "Embriogénese Somática - Pré-Requisito Para O Emprego de Algumas Técnicas de Biotecnologia No Melhoramento Genético de Plantas Perenes." *Biotecnologia Ciência & Desenvolvimento* 07: 36–39.

Becwar, M. R., R. Nagmani, and S. R. Wann. 1990. "Initiation of Embryogenic Cultures and Somatic Embryo Development in Loblolly Pine (*Pinus taeda*)." *Canadian Journal of Forest Research* 20 (6): 810–17. doi:10.1139/x90-107.

Benson, EA, K Harding, J Johnston, and JG Day. 2005. "From Ecosystem to Cryobanks the Role of Cryo-Conservation in the Preservation and Sustainable Utilization of Global Phyto-Diversity." In *Contributing to a Sustainable Future*, edited by I..J. Bennett, E. Bunn, H. Clarke, and J.A. McComb. Perth, Western Australia.

Benson, Erica E., Paul T. Lynch, and June Jones. 1992. "The Detection of Lipid Peroxidation Products in Cryoprotected and Frozen Rice Cells: Consequences for Post-Thaw Survival." *Plant Science* 85 (1): 107–14. doi:10.1016/0168-9452(92)90099-8.

Bonga, J. M., K. K. Klimaszewska, and P. von Aderkas. 2010. "Recalcitrance in Clonal Propagation, in Particular of Conifers." *Plant Cell, Tissue and Organ Culture (PCTOC)* 100 (3): 241–54. doi:10.1007/s11240-009-9647-2.

Briggs, Fred N., and Paulden Ford Knowles. 1967. *Introduction to Plant Breeding*. Reinhold Pub. Corp.

Bronson, M.R., and R.K. Dixon. 1991. "Cultural Factors Influencing Adventitious Shoot and Plantlet Formation from Slash Pine Cotyledons." *New Forests (Netherlands)* 5: 277–88.

Brune, A. 1982. "Estratégia Da Multiplicação Vegetativa No Melhoramento Florestal." *Revista Árvore* 6 (2): 162–65.

Burns, J A, O J Schwarz, and S E Schlarbaum. 1991. "Multiple Shoot Production from Seedling Explants of Slash Pine (*Pinus elliottii*, Engelm.)." *Plant Cell Reports* 10 (9): 439–43. doi:10.1007/BF00233810.

Cadenas, E. 1989. "Biochemistry of Oxygen Toxicity." *Annual Review of Biochemistry* 58 (January): 79–110. doi:10.1146/annurev.bi.58.070189.000455.

Calixto, Filomena, and M. Salomé Pais. 1997. "Adventitious Shoot Formation and Plant Regeneration from *Pinus pinaster* Sol. Ex Aiton." *In Vitro Cellular & Developmental Biology - Plant* 33 (2): 119–24. doi:10.1007/s11627-997-0008-8.

Carneros, E, C Celestino, K Klimaszewska, Y-S Park, M Toribio, and J M Bonga. 2009. "Plant Regeneration in Stone Pine (*Pinus pinea* L.) by Somatic Embryogenesis." *Plant Cell, Tissue and Organ Culture (PCTOC)* 98 (2): 165–78.

Choudhury, Hiranjit, Suman Kumaria, and Pramod Tandon. 2008. "Induction and Maturation of Somatic Embryos from Intact Megagametophyte Explants in Khasi Pine (*Pinus kesiya* Royle Ex. Gord.). Curr Sci." *Current Science* 95 (November): 1433–38.

Coke, J E. 1996. "Basal Nutrient Medium for in Vitro Cultures of Loblolly Pines." Google Patents.

Coppen, J. J. W., and G. A. Hone. 1995. *Gum Naval Stores: Turpentine and Rosin from Pine Resin*. Natural Resources Institute, FAO.

Cortizo, Millán, Nuria de Diego, Paloma Moncaleán, and Ricardo Javier Ordás. 2009.

"Micropropagation of Adult Stone Pine (*Pinus pinea* L.)." *Trees* 23 (4): 835–42. doi:10.1007/s00468-009-0325-0.

Crowe, J H, L M Crowe, J F Carpenter, A S Rudolph, C A Wistrom, B J Spargo, and T J Anchordoguy. 1988. "Interactions of Sugars with Membranes." *Biochimica et Biophysica Acta* 947 (2): 367–84.

Cyr, DR. 2000. "Cryopreservation: Roles in Clonal Propagation and Germplasm Conservation of Conifers." In *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application. Proceedings of an International Workshop*, edited by F Engelmann and H Takagi, 261–68. Tsukuba, Japan.

David, A, E Laine, and H David. 1995. "Somatic Embryogenesis in *Pinus caribaea*." In *Somatic Embryogenesis in Woody Plants*, 145–81. Springer.

De Diego, N., I. A. Montalbán, E. Fernandez de Larrinoa, and P. Moncaleán. 2008. "In Vitro Regeneration of *Pinus pinaster* Adult Trees." *Canadian Journal of Forest Research* 38 (10): 2607–15. doi:10.1139/X08-102.

De Diego, N., I.A. Montalbán, and P. Moncaleán. 2010. "In Vitro Regeneration of Adult *Pinus sylvestris* L. Trees." *South African Journal of Botany* 76 (1): 158–62. doi:10.1016/j.sajb.2009.09.007.

De Diego, Nuria, Itziar A. Montalbán, and Paloma Moncaleán. 2011. "Improved Micropropagation Protocol for Maritime Pine Using Zygotic Embryos." *Scandinavian Journal of Forest Research* 26 (3). Taylor & Francis Group: 202–11. doi:10.1080/02827581.2011.559174.

Debergh, P.C., and L.J. Maene. 1981. "A Scheme for Commercial Propagation of Ornamental Plants by Tissue Culture." *Scientia Horticulturae* 14 (4): 335–45. doi:10.1016/0304-4238(81)90047-9.

Dias, M. C., G. Pinto, C. M. Correia, J. Moutinho-Pereira, S. Silva, and C. Santos. 2013. "Photosynthetic Parameters of Ulmus Minor Plantlets Affected by Irradiance during Acclimatization." *Biologia Plantarum* 57 (1): 33–40. doi:10.1007/s10535-012-0234-8.

Dias, M. C., G. Pinto, and C. Santos. 2011. "Acclimatization of Micropropagated Plantlets Induces an Antioxidative Burst: A Case Study with Ulmus Minor Mill." *Photosynthetica* 49 (2): 259–66. doi:10.1007/s11099-011-0028-9.

Dieters, Mark, and Jeremy Brawner. 2007. "Productivity of *Pinus elliottii*, *P. caribaea* and Their F1 and F2 Hybrids to 15 Years in Queensland, Australia." *Annals of Forest Science* 64 (7): 691–98. doi:10.1051/forest:2007049.

Dieters, MJ, and DG Nikles. 1997. "The Genetic Improvement of Caribbean Pine (*Pinus caribaea* Morelet)—building on a Firm Foundation." In *Proc. 24th South. For. Tree Improv. Conf. Orlando FL*, 32–51.

Dodeman, V. L., G. Ducreux, and M. Kreis. 1997. "Zygotic Embryogenesis versus Somatic Embryogenesis. J Exp Bot." *Journal of Experimental Botany* 48 (313): 1493–1509.

Dumet, D., and E. E. Benson. 2000. "The Use of Physical and Biochemical Studies to Elucidate and Reduce Cryopreservation-Induced Damage in Hydrated/desiccated Plant Germplasm." In *Cryopreservation of Tropical Plant Germplasm*, edited by F. Engelmann and H. Takagi, 43–56. Tsukuba, Japan: International Plant Genetic Resources Institute (IPGRI).

Engelmann, F. 2000. "Importance of Cryopreservation for the Conservation of Plant Genetic Resources." In *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application. Proceedings of an International Workshop*, edited by F Engelmann and H Takagi, 8–20.

Engelmann, Florent. 2004. “Plant Cryopreservation: Progress and Prospects.” *In Vitro Cellular & Developmental Biology - Plant* 40 (5): 427–33. doi:10.1079/IVP2004541.

Faria, J. 2012. *Caderno Técnico - Produção Em Viveiros Florestais*. Edited by ANEFA. Lisboa: Effect.

Farjon, A. 1999. “World Checklist and Bibliography of Conifers.” *Nordic Journal of Botany* 19 (2): 148. doi:10.1111/j.1756-1051.1999.tb00657.x.

Farrar, J. L., and W. D. McJannet. 1959. “AIR-LAYERING OF EASTERN WHITE PINE (*Pinus strobus* L.).” *The Forestry Chronicle* 35 (1). NRC Research Press Ottawa, Canada: 19–21. doi:10.5558/tfc35019-1.

Floriano, EP. 2004b. “Produção de Mudas Por via Assexuada.” In *Produção de Sementes E Mudas Florestais*, edited by J.M. Hoppe, Caderno Di, 159–200. Universidade Federal de Santa Maria.

Foelkel, E. 2011. “Produção de Sementes Geneticamente Melhoradas de *Pinus*.” *Revista Da Madeira* 127.

Fonseca, Sebastião Machado da, and Paulo Yoshio Kageyama. 1978. “Melhoramento genético face à produção de resina.” Instituto de Pesquisas e Estudos Florestais.

Freitas, Miguel Luiz Menezes, Antonio Carlos Scatena Zanatto, Eurípedes Morais, Saulo Vannucci Lemos, Alessandro Chagas Fernandes, and Alexandre Magno Sebbenn. 2005. “Teste de Procedências de *Pinus caribaea* var. *hondurensis* Aos 32 Anos de Idade Em Bebedouro–SP.” *Revista Do Instituto Florestal* 17 (1): 17–23.

Furlan, Rodrigo de Andrade, Edson Seizo Mori, Evandro Vagner Tambarussi, Cristiano Bueno de Moraes, Frederico Almeida de Jesus, and Léo Zimback. 2007. “Estrutura genética de populações de melhoramento de *Pinus caribaea* var. *hondurensis* por meio de marcadores microssatélites.” *Bragantia* 66 (4). Bragantia: 553–63.

Gernandt, David S., Gretel Geada López, Sol Ortiz García, and Aaron Liston. 2005. “Phylogeny and Classification of *Pinus*.” In *Taxon*, 29–42. International Association for Plant Taxonomy.

Giri, C. C., B. Shyamkumar, and C. Anjaneyulu. 2004. “Progress in Tissue Culture, Genetic Transformation and Applications of Biotechnology to Trees: An Overview.” *Trees - Structure and Function* 18 (2): 115–35. doi:10.1007/s00468-003-0287-6.

Go, Naney E., Glorina D. Perez-Orozeo, and Saturnina C. Halos. 1993. “In Vitro Response of Embryos from Different Provenances of *Pinus caribaea* var. *hondurensis* Morelet.” *Plant Cell, Tissue and Organ Culture* 32 (1): 1–7. doi:10.1007/BF00040109.

Gomes, Pimentel. 1990. *Fruticultura Brasileira*. 11ª ed. São Paulo: NBL Editora.

Gresshoff, P M, and C H Doy. 1972. “Development and Differentiation of Haploid Lycopersicon Esculentum (tomato).” *Planta* 107 (2): 161–70. doi:10.1007/BF00387721.

Guerra, M.P., A.C. Torres, and J.B. Teixeira. 1999. “Embriogênese Somática E Sementes Sintéticas.” In *Culturas de Tecidos E Transformação Genética de Plantas*, edited by A. C. Torres, L.S. Caldas, and J.A. Buso, 533–68. Brasília: Embrapa-CBAB.

Gupta, P K, and D J Durzan. 1985. “Shoot Multiplication from Mature Trees of Douglas-Fir (*Pseudotsuga menziesii*) and Sugar Pine (*Pinus lambertiana*).” *Plant Cell Reports* 4 (4): 177–79. doi:10.1007/BF00269282.

Häggman, Hely M., Leena A. Ryynänen, Tuija S. Aronen, and Jana Krajnakova. 1998. “Cryopreservation of Embryogenic Cultures of Scots Pine.” *Plant Cell, Tissue and Organ Culture* 54 (1). Kluwer Academic Publishers: 45–53. doi:10.1023/A:1006104325426.

Halos, Saturnina C., and Nancy E. Go. 1993. "Micropropagation of *Pinus caribaea* Morelet." *Plant Cell, Tissue and Organ Culture* 32 (1): 47–53. doi:10.1007/BF00040115.

Harding, Keith, John G. Day, Maike Lorenz, Hella Timmermann, Thomas Friedl, David H. Bremner, and Erica E. Benson. 2004. "Introducing the Concept and Application of Vitrification for the Cryo-Conservation of Algae – a Mini-Review." *Nova Hedwigia* 79 (1): 207–26. doi:10.1127/0029-5035/2004/0079-0207.

Harding, Kevin J., and Terry R. Copley. 2000. "Wood Property Variation in Queensland-Grown Slash X Caribbean Pine Hybrids." In *Proceedings of Symposium on Hybrid Breeding and Genetics*, 7. Noosa: Queensland Forestry Research Institute.

Hargreaves, C, L Grace, and D Holden. 2002. "Nurse Culture for Efficient Recovery of Cryopreserved *Pinus radiata* D. Don Embryogenic Cell Lines." *Plant Cell Reports* 21 (1): 40–45. doi:10.1007/s00299-002-0478-4.

Hargreaves, C., and M. Menzies. 2007. "Organogenesis and Cryopreservation of Juvenile Radiata Pine." In *Protocols for Micropropagation of Woody Trees and Fruits*, edited by S. Mohan Jain and H. Häggman, 51–65. Dordrecht: Springer Netherlands. doi:10.1007/978-1-4020-6352-7.

Hargreaves, Cathy L., Cathie B. Reeves, Jens I. Find, Keiko Gough, Puthiyaparambil Josekutty, Dolina B. Skudder, Susan A. van der Maas, et al. 2009. "Improving Initiation, Genotype Capture, and Family Representation in Somatic Embryogenesis of *Pinus radiata* by a Combination of Zygotic Embryo Maturity, Media, and Explant Preparation." *Canadian Journal of Forest Research* 39 (8): 1566–74. doi:10.1139/X09-082.

Hartmann, H. T., and D. E. Kester. 1975. *Plant Propagation: Principles and Practices.* 3.Ed. ed. New Jersey: Prentice-Hall.

Hazubska-Przybył, Teresa, Paweł Chmielarz, Marcin Michalak, and Krystyna Bojarczuk. 2010. "Cryopreservation of Embryogenic Tissues of *Picea omorika* (Serbian Spruce)." *Plant Cell, Tissue and Organ Culture (PCTOC)* 102 (1): 35–44. doi:10.1007/s11240-010-9701-0.

Higa, AR. 2002. "Melhoramento." *Revista Da Madeira* n°68.

Higashi, EN, and RLV de Arruda Silveira. 2004. "Fertirrigação Em Viveiros de Mudas de *Eucalyptus* E *Pinus*." In . Piracicaba - SP - Brasil: RR Agroflorestal S/C Ltda.

Higashi, Katsumi, Hiroshi Kamada, and Hiroshi Harada. 1996. "The Effects of Reduced Nitrogenous Compounds Suggests That Glutamine Synthetase Activity Is Involved in the Development of Somatic Embryos in Carrot." *Plant Cell, Tissue and Organ Culture* 45 (2): 109–14. doi:10.1007/BF00048752.

Humánez, A., M. Blasco, C. Brisa, J. Segura, and I. Arrillaga. 2012. "Somatic Embryogenesis from Different Tissues of Spanish Populations of Maritime Pine." *Plant Cell, Tissue and Organ Culture (PCTOC)* 111 (3): 373–83. doi:10.1007/s11240-012-0203-0.

Humánez, Alicia, Miquel Blasco, Carmen Brisa, Juan Segura, and Isabel Arrillaga. 2011. "Thidiazuron Enhances Axillary and Adventitious Shoot Proliferation in Juvenile Explants of Mediterranean Provenances of Maritime Pine *Pinus pinaster*." *In Vitro Cellular & Developmental Biology - Plant* 47 (5): 569–77. doi:10.1007/s11627-011-9397-9.

Jones, N.B., and J. Van Staden. 1995. "Plantlet Production from Somatic Embryos of *Pinus patula*." *Journal of Plant Physiology* 145 (4): 519–25. doi:10.1016/S0176-1617(11)81781-5.

Jones, Nicky B., and Johannes van Staden. 2001. "Improved Somatic Embryo Production from Embryogenic Tissue of *Pinus patula*." *In Vitro Cellular & Developmental Biology - Plant* 37 (5): 543–49. doi:10.1007/s11627-001-0094-y.

Kaczmarczyk, Anja, Bryn Funnekotter, Akshay Menon, Pui Ye Phang, Arwa Al-Hanbali, Eric Bunn, and Ricardo L. Mancera. 2012. *Current Frontiers in Cryobiology*. Edited by Igor Katkov. *Current Frontiers in Cryobiology*. InTech. doi:10.5772/1962.

Kalia, R. K., S. Arya, S. Kalia, and I. D. Arya. 2007. "Plantlet Regeneration from Fascicular Buds of Seedling Shoot Apices of *Pinus roxburghii* Sarg." *Biologia Plantarum* 51 (4): 653–59. doi:10.1007/s10535-007-0138-1.

Kalita, V, H Choudhury, S Kumaria, and P Tandon. 2012. "Vitrification-Based Cryopreservation of Shoot-Tips of *Pinus kesiya* Royle Ex. Gord." *Cryo Letters* 33 (1): 58–68.

Kami, Daisuke. 2012. "Current Frontiers in Cryobiology." In *Current Frontiers in Cryobiology*, edited by Igor Katkov, 439–56. InTech. doi:10.5772/1962.

Kerbauy, G.B. 1997. "Clonagem de Plantas in Vitro." *Revista Biotecnologia, Ciência E Desenvolvimento* 1 (1): 30–33.

Kim, YW, and HK Moon. 2007. "Regeneration of Plant by Somatic Embryogenesis in *Pinus rigida* × *P. taeda*." *In Vitro Cellular & Developmental Biology-Plant* 43 (4): 335–42. doi:10.1007/s116727-007-9045-6.

Klimaszewska, K., C. Noceda, G. Pelletier, P. Label, R. Rodriguez, and M. A. Lelu-Walter. 2009. "Biological Characterization of Young and Aged Embryogenic Cultures of *Pinus pinaster* (Ait.)." *In Vitro Cellular & Developmental Biology - Plant* 45 (1): 20–33. doi:10.1007/s11627-008-9158-6.

Klimaszewska, K., & Smith, D. R. (1997). Maturation of somatic embryos of *Pinus* strobus is promoted by a high concentration of gellan gum. Physiologia Plantarum, 100(4), 949–957.

Klimaszewska, K., J.F. Trontin, M.R. Becwar, C Devillard, Y.S. Park, and M.A. Lelu-Walter. 2007. "Recent Progress in Somatic Embryogenesis of Four *Pinus* Spp." *Tree and Forestry Science and Biotechnology* 1: 11–25.

Klimaszewska, Krystyna, Yill-Sung Park, Cathy Overton, Ian Maceacheron, and Jan M. Bonga. 2001. "Optimized Somatic Embryogenesis in *Pinus strobus* L." *In Vitro Cellular & Developmental Biology - Plant* 37 (3): 392–99. doi:10.1007/s11627-001-0069-z.

Langis, R., B. Schnabel, E.D. Earle, and P.L. Steponkus. 1989. "Cryopreservation of Brassica Campestris L. Cell Suspensions by Vitrification." *Cryo Letters* 10: 421–28.

Lara-Chavez, Alejandra, Barry S Flinn, and Ulrika Egertsdotter. 2011. "Initiation of Somatic Embryogenesis from Immature Zygotic Embryos of Oocarpa Pine (*Pinus oocarpa* Schiede Ex Schlectendal)." *Tree Physiology* 31 (5): 539–54. doi:10.1093/treephys/tpr040.

Latutrie, Mathieu, and Tuija Aronen. 2013. "Long-Term Cryopreservation of Embryogenic *Pinus sylvestris* Cultures." *Scandinavian Journal of Forest Research* 28 (2). Taylor & Francis Group: 103–9. doi:10.1080/02827581.2012.701325.

Lelu-Walter, MA, M Bernier-Cardou, and K Klimaszewska. 2006. "Simplified and Improved Somatic Embryogenesis for Clonal Propagation of *Pinus pinaster* (Ait.)." *Plant Cell Reports* 25 (8): 767–76. doi:10.1007/s00299-006-0115-8.

Li, Xin Y., Feng H. Huang, and Edward E. Gbur. 1997. "Polyethylene Glycol-Promoted Development of Somatic Embryos in Loblolly Pine (*Pinus taeda* L.)." *In Vitro Cellular & Developmental Biology - Plant* 33 (3): 184–89. doi:10.1007/s11627-997-0019-5.

Liao, Y. K., and H. V. Amerson. 1995. "Slash Pine (*Pinus elliottii* Engelm.) Somatic Embryogenesis I. Initiation of Embryogenic Cultures from Immature Zygotic Embryos." *New Forests* 10 (2). Kluwer Academic Publishers: 145–63. doi:10.1007/BF00033404.

Lipavská, Helena, and Hana Konrádová. 2004. "Somatic Embryogenesis in Conifers: The Role of Carbohydrate Metabolism." *In Vitro Cellular & Developmental Biology - Plant* 40 (1): 23–30. doi:10.1079/IVP2003482.

Litvay, J D, D C Verma, and M A Johnson. 1985. "Influence of a Loblolly Pine (*Pinus taeda* L.). Culture Medium and Its Components on Growth and Somatic Embryogenesis of the Wild Carrot (*Daucus carota* L.)." *Plant Cell Reports* 4 (6): 325–28. doi:10.1007/BF00269890.

Lohrey, R.E., and S.V. Kossuth. 1990. "*Pinus elliottii* Engelm." In *Silvics of North America. Agriculture Handbook 654*, edited by R.M. Burns and B.H. Honkala. Washington, DC.

López, Marián, José Pacheco, Roberto Rodríguez, and Ricardo J. Ordás. 1996. "Regeneration of Plants from Isolated Cotyledons of Salgareño Pine (*Pinus nigra* Arn. ssp.Salzmannii (Dunal) Franco)." *In Vitro Cellular & Developmental Biology - Plant* 32 (2): 109–14. doi:10.1007/BF02823140.

LoSchiavo, F., L Pitto, G Giuliano, G Torti, V Nuti-Ronchi, D Marazziti, R Vergara, S Orselli, and M Terzi. 1989. "DNA Methylation of Embryogenic Carrot Cell Cultures and Its Variations as Caused by Mutation, Differentiation, Hormones and Hypomethylating Drugs." *Theoretical and Applied Genetics* 77 (3): 325–31. doi:10.1007/BF00305823.

Lv, Chengqun, and Baoling Huang. 2012. "Stem Tissue Culture of *Pinus elliottii* × *Pinus caribaea*." In *2012 International Conference on Biomedical Engineering and Biotechnology*, 1700–1703. IEEE. doi:10.1109/iCBEB.2012.366.

Lynch, Paul T., Erica E. Benson, and Harding Keith. 2007. "Climate Change: The Role of Ex Situ and Cryo-Conservation in the Future Security of Economically Important, Vegetatively Propagated Plants." *The Journal of Horticultural Science and Biotechnology* 82 (2). Taylor & Francis: 157–60.

Malabadi, Ravindra B, and Johannes Van Staden. 2005. "Somatic Embryogenesis from Vegetative Shoot Apices of Mature Trees of *Pinus patula*." *Tree Physiology* 25 (1): 11–16.

Malabadi, Ravindra B., and K. Nataraja. 2006. "Cryopreservation and Plant Regeneration via Somatic Embryogenesis Using Shoot Apical Domes of Mature *Pinus roxburghii* Sarg, Trees." *In Vitro Cellular & Developmental Biology - Plant* 42 (2): 152–59. doi:10.1079/IVP2005731.

Mantell, S.H., J.A. Matthews, and R.A. McKEE. 1994. "Principios de Biotecnologia Em Plantas: Uma Introducao a Engenharia Genetica Em Plantas." *Ribeirao Preto: Revista Brasileira de Genetica*.

Martinez-Montero, ME, and K Harding. 2015. "Cryobionomics: Evaluating the Concept in Plant Cryopreservation." In *PlantOmics: The Omics of Plant Science*, edited by Debmalya Barh, Muhammad Sarwar Khan, and Eric Davies, 655–82. New Delhi: Springer India. doi:10.1007/978-81-322-2172-2.

Marum, Liliana, Catarina Estêvão, M. Margarida Oliveira, Sara Amâncio, Lucília Rodrigues, and Célia Miguel. 2004. "Recovery of Cryopreserved Embryogenic Cultures of Maritime Pine - Effect of Cryoprotectant and Suspension Density." *Cryoletters* 25 (5). Cryoletters: 363–74.

Maruyama, Emilio, Yoshihisa Hosoi, and Katsuaki Ishii. 2007. "Somatic Embryogenesis and Plant Regeneration in Yakutanegoyou, *Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima, an Endemic and Endangered Species in Japan." *In Vitro Cellular & Developmental Biology - Plant* 43 (1): 28–34. doi:10.1007/s11627-006-9003-8.

Mathur, G., V.A. Alkutkar, and R.S. Nadgauda. 2003. "Cryopreservation of Embryogenic Culture of *Pinus roxburghii*." *Biologia Plantarum* 46 (2). Kluwer Academic Publishers: 205–10. doi:10.1023/A:1022894409408.

Mathur, G., and R. Nadgauda. 1999. “In Vitro Plantlet Regeneration from Mature Zygotic Embryos of *Pinus wallichiana* A.B. Jacks.” *Plant Cell Reports* 19 (1): 74–80. doi:10.1007/s002990050713.

Medrado, M.J.S. 2011. “Cultivo de *Pinus* - Guia de Orientações Básicas Sobre Cultivo.” Embrapa Florestas.

Meryman, H T, R J Williams, and M S Douglas. 1977. “Freezing Injury from ‘Solution Effects’ and Its Prevention by Natural or Artificial Cryoprotection.” *Cryobiology* 14 (3): 287–302.

Miguel, Célia, Sónia Gonçalves, Susana Tereso, Liliana Marum, João Maroco, and M. Margarida Oliveira. 2004. “Somatic Embryogenesis from 20 Open-Pollinated Families of Portuguese Plus Trees of Maritime Pine.” *Plant Cell, Tissue and Organ Culture* 76 (2): 121–30. doi:10.1023/B:TICU.0000007253.91771.e3.

Miguel, Célia, and Liliana Marum. 2011. “An Epigenetic View of Plant Cells Cultured in Vitro: Somaclonal Variation and beyond.” *Journal of Experimental Botany* 62 (11): 3713–25. doi:10.1093/jxb/err155.

Misra, Santosh. 1994. “Conifer Zygotic Embryogenesis, Somatic Embryogenesis, and Seed Germination: Biochemical and Molecular Advances.” *Seed Science Research* 4 (04): 357–84. doi:10.1017/S0960258500002439.

Montalban, I. A., N. De Diego, and P. Moncalean. 2011. “Testing Novel Cytokinins for Improved in Vitro Adventitious Shoots Formation and Subsequent Ex Vitro Performance in *Pinus radiata*.” *Forestry* 84 (4): 363–73. doi:10.1093/forestry/cpr022.

Montalbán, I. A., A. Setién-Olarra, C. L. Hargreaves, and P. Moncaleán. 2013. “Somatic Embryogenesis in *Pinus halepensis* Mill.: An Important Ecological Species from the Mediterranean Forest.” *Trees* 27 (5): 1339–51. doi:10.1007/s00468-013-0882-0.

Montalbán, IA, N De Diego, and P Moncaleán. 2010. “Bottlenecks in *Pinus radiata* Somatic Embryogenesis: Improving Maturation and Germination.” *Trees* 24 (6): 1061–71. doi:10.1007/s00468-010-0477-y.

Muniz, Aleksander Westphal, Enilson Luiz de Sá, Gilberto Luíz Dalagnol, and João Américo Filho. 2013. “Rooting and Acclimatization of Micropropagated Marubakaido Apple Rootstock Using Adesmia Latifolia Rhizobia.” *SpringerPlus* 2 (January): 437. doi:10.1186/2193-1801-2-437.

Murashige, T, and F Skoog. 1962. “A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures.” *Physiologia Plantarum* 15 (3): 473–97. doi:10.1111/j.1399-3054.1962.tb08052.x.

Namkoong, Gene, Hyun Chung Kang, and Jean Sébastien Brouard. 1988. *Tree Breeding: Principles and Strategies*. Vol. 11. Monographs on Theoretical and Applied Genetics. New York, NY: Springer New York. doi:10.1007/978-1-4612-3892-8.

Nandwani, D, S Kumaria, and P Tandon. 2001. “Micropropagation of *Pinus kesiya* Royle Ex Gord (Khasi Pine).” *Gartenbauwissenschaft* 66 (2): 68–71.

Netto, D.A.M. 2010. “Coleção de base e coleção ativa: o banco de germoplasma de sorgo.” Sete Lagoas: Embrapa Milho e Sorgo.

Newton, RJ, KA Marek-Swize, ME Magallanes-Cedeno, N Dong, S Sen, and SM Jain. 1995. “Somatic Embryogenesis in Slash Pine (*Pinus elliottii* Engelm.).” In *Somatic Embryogenesis in Woody Plants, Volume 3 - Gymnosperms*, edited by SM Jain, PK Gupta, and RJ Newton, 183–95. doi:10.1007/978-94-011-0960-4_11.

Newton, RJ, W Tang, and SM Jain. 2005. "SLASH PINE (*Pinus elliottii* Engelm.)." In *Protocol for Somatic Embryogenesis in Woody Plants*, 1–10. Springer Science & Business Media.

Nikles, DG. 2000. "Experience with Some *Pinus* Hybrids in Queensland, Australia." In *Proceedings of QFRI/CRC-SPF Symposium: Hybrid Breeding and Genetics of Forest Trees*, edited by HS Dungey, MJ Dieters, and DG Nikles, 27–43. Noosa, Queensland, Australia: Department of Primary Industries, Brisbane.

Nikles, DG, and MJ Robinson. 1989. "The Development of *Pinus* Hybrids for Operational Use in Queensland." In *Proceedings of IUFRO Conference: Breeding Tropical Trees*, 25–35. Pattaya.

Oliveira, Leandro Francisco de. 2011. "Micropropagação de *Pinus taeda* L. a partir de material juvenil." Universidade Federal do Paraná, Curitiba.

Oliveira, LF, LLF Ribas, M Quoirin, HS Koehler, E Amano, and AR Higa. 2012. "Micropropagation of *Pinus taeda* L. from Juvenile Material." *Tree and Forestry Science and Biotechnology* 6 (1): 96–101.

Panis, Bart, and M Lambardi. 2005. "Status of Cryopreservation Technologies in Plants (crops and Forest Trees)." In *International Workshop on "The Role of Biotechnology for the Characterisation and Conservation of Crop, Forestry, Animal and Fishery Genetic Resources,"* 43–54.

Park, Y. S., J. D. Barrett, and J. M. Bonga. 1998. "Application of Somatic Embryogenesis in High-Value Clonal Forestry: Deployment, Genetic Control, and Stability of Cryopreserved Clones." *In Vitro Cellular & Developmental Biology - Plant* 34 (3): 231–39. doi:10.1007/BF02822713.

Park, Y. S., M. A. Lelu-Walter, L. Harvengt, J. F. Trontin, I. MacEacheron, K. Klimaszewska, and J. M. Bonga. 2006. "Initiation of Somatic Embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at Three Laboratories in Canada and France." *Plant Cell, Tissue and Organ Culture* 86 (1): 87–101. doi:10.1007/s11240-006-9101-7.

Park, Y.S. 2014. "Conifer Somatic Embryogenesis and Multi-Varietal Forestry." In *Challenges and Opportunities for the World's Forests in the 21st Century*, edited by Trevor Fenning, 81:425–39. Forestry Sciences. Dordrecht: Springer Netherlands. doi:10.1007/978-94-007-7076-8.

Park, YS, and J Bonga. 2011. "Applications of Somatic Embryogenesis in Forest Management and Research." In *Proceedings of the IUFRO Working Party 2.09.02*, 3–9.

Pascoe, S. 2002. "Somatic Embryogenesis In *Pinus radiata*." Faculty of Engineering and Science, Victoria University.

Pinus Brasil. 2007. "Hibrido de *Pinus* Eliliondurensis." http://pinusbrasil.com.br/site/br/.

Pullman, G S, S Johnson, G Peter, J Cairney, and N Xu. 2003. "Improving Loblolly Pine Somatic Embryo Maturation: Comparison of Somatic and Zygotic Embryo Morphology, Germination, and Gene Expression." *Plant Cell Reports* 21 (8): 747–58. doi:10.1007/s00299-003-0586-9.

Pullman, Gerald S, and Kylie Bucalo. 2011. "Pine Somatic Embryogenesis Using Zygotic Embryos as Explants." In *Methods in Molecular Biology (Clifton, N.J.)*, edited by TA Thorpe and EC Yeung, 710:267–91. Springer Science+Business Media. doi:10.1007/978-1-61737-988-8_19.

Pullman, Gerald S., and Shannon Johnson. 2002. "Somatic Embryogenesis in Loblolly Pine (*Pinus taeda* L.): Improving Culture Initiation Rates." *Annals of Forest Science* 59 (5-6). EDP Sciences: 663–68. doi:10.1051/forest:2002053.

Pullman, GS, J Mein, S Johnson, and Y Zhang. 2005. "Gibberellin Inhibitors Improve

Embryogenic Tissue Initiation in Conifers." *Plant Cell Reports* 23 (9): 596–605. doi:10.1007/s00299-004-0880-1.

Ragonezi, Carla, Krystyna Klimaszewska, Mário Rui Castro, Mónica Lima, Paulo de Oliveira, and Maria Amely Zavattieri. 2010. "Adventitious Rooting of Conifers: Influence of Physical and Chemical Factors." *Trees* 24 (6): 975–92. doi:10.1007/s00468-010-0488-8.

Rall, W F, and G M Fahy. 1985. "Ice-Free Cryopreservation of Mouse Embryos at -196 Degrees C by Vitrification." *Nature* 313 (6003): 573–75.

Reed, B. M., and Y. Chang. 1997. "Medium- and Long-Term Storage of in Vitro Cultures of Temperate Fruit and Nut Crops." In *Conservation of Plant Genetic Resources In Vitro*, edited by M.K. Razdan and E.C. Cocking, 67–105. Enfield: Science Publishers.

Reinert, J. 1958. "Untersuchungen über Die Morphogenese an Gewebekulturen." *Ber. Dtsch. Bot. Ges.* 71: 15.

Reinhoud, PJ, F van Iren, and JW Kijne. 2000. "Cryopreservation of Undifferentiated Plant Cells." In *Cryopreservation of Tropical Plant Germplasm*, 91–102.

Resende, M.D.V. 1999. "Melhoramento de Essências Florestais." In *Melhoramento de Espécies Cultivadas*, edited by A. Borém, 589–647. Viçosa: MG: Ed. da UFV.

Resende, M.D.V., and M.H.P. Barbosa. 2005. *Melhoramento Genético de Plantas de Propagação Assexuada*. Colombo: Embrapa Florestas.

Rodrigues, Kelly Cristine da Silva. 2006. "Caracterização e otimização da produção de resina em *Pinus elliottii* Engelm. - Papel de moduladores bioquímicos." Universidade Federal do Rio Grande do Sul.

Rodríguez, R., M. Fernández, J. Pacheco, and M.J. Cañal. 2005. "Envejecimiento Vegetal, Una Barrera a La Propagación. Alternativas." In *Biotecnologia Foretal En Especies Leñosas de Interés Forestall*, edited by M.E.S. Olate and D.G.R. Leal, 29–48. Concepción.

Saborio, F., W. S. Dvorak, J. K. Donahue, and T. A. Thorpe. 1997. "In Vitro Regeneration of Plantlets from Mature Embryos of *Pinus ayacahuite*." *Tree Physiology* 17 (12): 787–96. doi:10.1093/treephys/17.12.787.

Salaj, T., I. Matušíková, L. Fráterová, B. Piršelová, and J. Salaj. 2011. "Regrowth of Embryogenic Tissues of *Pinus nigra* Following Cryopreservation." *Plant Cell, Tissue and Organ Culture (PCTOC)* 106 (1): 55–61. doi:10.1007/s11240-010-9893-3.

Salajova, T, and J Salaj. 2005. "Somatic Embryogenesis in *Pinus nigra*: Embryogenic Tissue Initiation, Maturation and Regeneration Ability of Established Cell Lines." *Biologia Plantarum* 49 (3): 333–39. doi:10.1007/s10535-005-0003-z.

Shimizu, J. Y., and I. H. Z. Spir. 1999. "Seleção de *Pinus elliottii* pelo valor genético para alta produção de resina." *Boletim de Pesquisa Florestal* 38. Boletim de Pesquisa Florestal, Colombo: 103–17.

Simão, S. 1998. *Tratado de Fruticultura*. Piracicaba: FEALQ.

Simione, F.P. 1998. *Cryopreservation Manual Nalge Nunc International*. American Type Culture Collection (ATCC) in cooperation with Nalge Nunc International.

Smith, DR, A Warr, L Grace, C Walter, and C Hargreaves. 1994. "Somatic Embryogenesis Joins the Plantation Forestry Revolution in New Zealand." In *Proceedings of TAPPI 1994 Biological Sciences Symposium*, 19–29. Minneapolis.

Steward, F. C., Marion O. Mapes, and Kathryn Mears. 1958. "Growth and Organized Development

of Cultured Cells. II. Organization in Cultures Grown from Freely Suspended Cells." *American Journal of Botany* 45 (10): 705–8. doi:10.2307/2439728.

Stojičić, Dragana, Branka Uzelac, Dušica Janošević, Ljubinka Ćulafić, and Snežana Budimir. 2007. "Induction of Somatic Embryogenesis in *Pinus heldreichii* Culture." *Archives of Biological Sciences* 59 (3): 199–202. doi:10.2298/ABS0703199S.

Sudarmonowati, E., F. Engelmann, and H. Takagi. 2000. "Cryopreservation of Tropical Plants: Current Research Status in Indonesia." In *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application.*, 291–96. International Plant Genetic Resources Institute (IPGRI).

Takagi, H. 2000. "Recent Developments in Cryopreservation of Shoot Apices of Tropical Species." In *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application.*, edited by H. Takagi and F. Engelmann, 178–93. International Plant Genetic Resources Institute (IPGRI).

Tang, W, Z Guo, and F Ouyang. 2001. "Plant Regeneration from Embryogenic Cultures Initiated from Mature Loblolly Pine Zygotic Embryos." *In Vitro Cellular & Developmental Biology-Plant* 37 (5): 558–63. doi:10.1007/s11627-001-0097-8.

Tang, W, and RJ Newton. 2007. "Protocols for Micropropagation of Woody Trees and Fruits." In *Protocols for Micropropagation of Woody Trees and Fruits*, edited by S. Mohan Jain and H. Häggman, 15–22. Dordrecht: Springer Netherlands. doi:10.1007/978-1-4020-6352-7.

Tang, Wei, Ronald J Newton, and Thomas M Charles. 2006. "Plant Regeneration through Multiple Adventitious Shoot Differentiation from Callus Cultures of Slash Pine (*Pinus elliottii*)." *Journal of Plant Physiology* 163 (1): 98–101. doi:10.1016/j.jplph.2005.04.030.

Teasdale, R D, P A Dawson, and H W Woolhouse. 1986. "Mineral Nutrient Requirements of a Loblolly Pine (*Pinus taeda*) Cell Suspension Culture : Evaluation of a Medium Formulated from Seed Composition Data." *Plant Physiology* 82 (4): 942–45.

Tereso, S, S Gonçalves, L Marum, M Oliveira, J Maroco, and C Miguel. 2006. "Improved Axillary and Adventitious Bud Regeneration from Portuguese Genotypes of *Pinus pinaster* Ait." *Propagation of Ornamental Plants* 6 (1): 24–33.

Torres, A. C., A.T. Ferreira, F. G. de Sá, J. A. Buso, L. S. Caldas, A. S. Nascimento, M. de M. Brigido, and E. Romano. 2000. "Glossario de biotecnologia vegetal." Brasilia: Embrapa Hortalicas.

Uragami, A, A Sakai, M Nagai, and T Takahashi. 1989. "Survival of Cultured Cells and Somatic Embryos of Asparagus Officinalis Cryopreserved by Vitrification." *Plant Cell Reports* 8 (7): 418–21. doi:10.1007/BF00270083.

Valledor, L., R. Rodríguez, P. Sánchez, M.F. Fraga, M. Berdasco, R. Hasbún, J.L Rodriguez, et al. 2007. "Propagation of Selected *Pinus* Genotypes regardless of Age." In *Protocols for Micropropagation of Woody Trees and Fruits*, edited by S. Mohan Jain and H. Häggman, 137–46. Dordrecht: Springer Netherlands. doi:10.1007/978-1-4020-6352-7.

von Arnold, Sara, Izabela Sabala, Peter Bozhkov, Julia Dyachok, and Lada Filonova. 2002. "Developmental Pathways of Somatic Embryogenesis." *Plant Cell, Tissue and Organ Culture* 69 (3). Kluwer Academic Publishers: 233–49. doi:10.1023/A:1015673200621.

Walter, C., J.I. Find, and J. Grace. 1998. "Somatic Embryogenesis and Genetic Transformation in *Pinus radiata*." In *Somatic Embryogenesis in Woody Plants - Vol.4*, edited by S.M. Jain, P.K. Gupta, and R.J. Newton, 491–504. Kluwer Academic Publishers.

Walter, C. 2005. "Somatic Embryogenesis and Genetic Transformation in *Pinus radiata*." In

Protocol for Somatic Embryogenesis in Woody Plants, edited by S. Mohan Jain and Pramod K. Gupta, 77:11–24. Forestry Sciences. Dordrecht: Springer Netherlands. doi:10.1007/1-4020-2985-3.

Wendling, I. 2003. "Propagação Vegetativa." In *Semana Do Estudante Universitário-2003 Florestas E Meio Ambiente*. Colombo: Embrapa Florestas.

White, TL, and TD Byram. 2004. "Slash Pine Tree Improvement." In *Slash Pine: Still Growing and Growing! Proceedings of the Slash Pine Symposium*, edited by E.D. Dickens, J.P. Barnett, W.G. Hubbard, and E.J. Jokela. Asheville, NC: U.S: Department of Agriculture, Forest Service, Southern Research Station.

Williams, Claire G. 2009. *Conifer Reproductive Biology*. Dordrecht: Springer Netherlands. doi:10.1007/978-1-4020-9602-0.

Williams, R J, and A C Leopold. 1989. "The Glassy State in Corn Embryos." *Plant Physiology* 89 (3): 977–81.

Withers, LA, and PJ King. 1980. "A Simple Freezing Unit and Routine Cryopreservation Method for Plant-Cell Cultures." *Cryo-Letters* 1: 213–20.

Xavier, A. 2002. *Silvicultura Clonal I: Princípios E Técnicas de Propagação Vegetativa. (Caderno Didático)*. *Universidade Federal de Viçosa*. Viçosa.

Yeung, E.C. 1995. "Structural and Development Patterns in Somatic Embryogenesis." In *In Vitro Embryogenesis in Plants*, 205–47. Springer Science & Business Media.

Yildirim, Tolga, Zeki Kaya, and Kani Işik. 2006. "Induction of Embryogenic Tissue and Maturation of Somatic Embryos in *Pinus brutia* TEN." *Plant Cell, Tissue and Organ Culture* 87 (1): 67–76. doi:10.1007/s11240-006-9137-8.

Zámečník, Jiří, Miloš Faltus, Alois Bilavčík, and Renata Kotková. 2012. "Comparison of Cryopreservation Methods of Vegetatively Propagated Crops Based on Thermal Analysis." In *Current Frontiers in Cryopreservation*, edited by Igor Katkov, 333–58. InTech. doi:10.5772/2528.

Zavattieri, A, M Lima, V Sobral, P Oliveira, and A Costa. 2009. "Effects of Carbon Source, Carbon Concentration and Culture Conditions on in Vitro Rooting of *Pinus pinea* L. Microshoots." *Acta Hort (ISHS)* 812: 173–80.

Žel, J., N. Gogala, and M. Camloh. 1988. "Micropropagation of *Pinus sylvestris*." *Plant Cell, Tissue and Organ Culture* 14 (3): 169–75. doi:10.1007/BF00043407.

Zhang, Cun-Xu, Qian Li, and Lisheng Kong. 2007. "Induction, Development and Maturation of Somatic Embryos in Bunge's Pine (*Pinus bungeana* Zucc. Ex Endl.)." *Plant Cell, Tissue and Organ Culture* 91 (3): 273–80. doi:10.1007/s11240-007-9294-4.

Zhu, Li-Hua, Xiao-Qin Wu, Hong-Ye Qu, Jing Ji, and Jian-ren Ye. 2010. "Micropropagation of *Pinus massoniana* and Mycorrhiza Formation in Vitro." *Plant Cell, Tissue and Organ Culture (PCTOC)* 102 (1): 121–28. doi:10.1007/s11240-010-9711-y.

Zobel, B.Z., and J.A. Talbert. 1984. "Applied Forest Tree Improvement," January.

CHAPTER II

MICROPROPAGATION OF *PINUS ELLIOTTII* VAR. *ELLIOTTII*

II.1 EFFICIENT PROTOCOL FOR *IN VITRO* MASS MICROPROPAGATION OF SLASH PINE

Chapter prepared to be submitted as an original paper to a SCI journal:

Nunes S, Sousa D, Dias MC, Pereira VT, Correia S, Marum L, Santos C. 2016. Efficient protocol for *in vitro* mass micropropagation o Slash Pine.

Abstract

A protocol was developed for large-scale micropropagation of Slash Pine (*Pinus elliottii* var. *elliottii*). For that, first a seed disinfection and germination protocol was optimized allowing a routine (~70%) production of seedlings. Then, explants consisting of shoot apices 1.5 – 2.0 cm in length from four week-old seedlings were cultured on three different basal media (MS, GD and WV5). All media were supplemented with 6-benzylaminopurine (BAP), to stimulate the formation of axillary buds. Best bud induction was achieved, after four weeks, on WV5 medium (with 10 μM BAP) with rates close to 100%, and an average number of 7 new buds formed per explant. Elongation took place for six weeks on WV5 medium containing activated charcoal (0.2% w/v) and without growth regulators (GR). Rooting took place on half-strength WV5 medium containing 2 $mg.L^{-1}$ indole-3-butyric acid (IBA). After six weeks root primordia were visible in ~44% of slash pine shoots. The acclimatization protocol was also optimized by controlling relative humidity, light/photoperiod, temperature and nutrition, which led to an acclimatization success of ~90%. True-to-typeness of micropropagated plants was demonstrated by comparing by flow cytometry the DNA-ploidy of cloned plantlets with that of seedlings. With the protocol here described it is possible to obtain genetically uniform plants 20 to 22 weeks after *in vitro* germination of slash pine seeds.

Key words: slash pine, *Pinus elliottii*, micropropagation, *Pinus* true-to-typeness

Introduction

Pines (*Pinus* sp.) are amongst the most important conifer trees in the supply of the forestry-wood chain throughout the world. In particular, industrial plantations of slash pine (*Pinus elliottii* var. *elliottii*) have been established worldwide (eg., Brazil, USA, Australia) with enormous economic value, as raw material for wood and resin industry. *P. elliottii* plants reach commercialy valuable wood sooner than other *Pinus* species, and have multiple uses namely as wood and resin, or even as source for cellulose. The domestication of forest trees for industrial purposes (eg., resin, fiber, wood) began some decades ago, and is now benefiting from biotechnological tools to clone elite genotypes and or genetically improve for desirable traits (e.g. Campbell et al. 2003). Clonal propagation of forest species has significant advantages for the mass propagation of selected genotypes and is used in breeding strategies to produce improved plant stocks more rapidly than conventional seed orchard procedures (Tereso et al. 2006; Pinto et al. 2008). However, breeding programs are often constrained by limited knowledge of genetic variation, a long period of reproductive maturity in forest species, recurrent difficulties in storing seed for long periods, and frequent recalcitrance in vegetative propagation (Pinto et al. 2002). Tree species micropropagation is an effective way of capturing genetic gain with the potential to provide very high multiplication rates of selected tree genotypes, resulting in short-term silviculture gains (Oliveira et al. 2012). Moreover, it overcomes problems of irregular seed cone production, long life cycles and recalcitrance of conventional vegetative propagation methods. However, the commercial use of micropropagation is limited in many woody species due to the high variability on the efficiency of adventitious rooting (Kalia et al. 2007), or to the poor plantlet survival rates during the acclimatization process (e.g. Loureiro et al. 2007a).

Technological approaches have been applied to both improve and clone pine selected germoplasms (e.g. Campbell et al. 2003). Micropropagation of *Pinus* have been already reported for several species from juvenile material (e.g. zygotic embryos, nodal and apical segments of seedlings) of *P. pinaster* (Azevedo et al. 2001; Tereso et al. 2006), *P. kesiya* (Nandwani et al. 2001), *P. roxburghii* (Kalia et al. 2007), or *P. taeda* (Oliveira et al. 2012). Shoot bud segments were also obtained from adult material from *P. pinea* (Cortizo et al. 2009), *P. pinaster* (De Diego et al. 2008), *P. sylvestris* (De Diego et al. 2010) and *P. brutia*

Ten. (Abdullah et al. 1987). This micropropagation strategy is a simple technique compared to others, first because it uses preformed meristems but also because it allows greater genetic stability, less somaclonal variation and also avoids using high concentrations of cytokinins for the development of axillary buds (e.g. Oliveira et al. 2012). The cytokinin 6-benzyladenine (BAP) seems to be highly beneficial for successufully induce axillary shoots in most of the protocols proposed for the studied *Pinus* species (e.g. Calixto & Pais 1997; Saborio et al. 1997; Azevedo et al. 2001; Zhang et al. 2003; Alonso et al. 2006; Tereso et al. 2006; Kalia et al. 2007; De Diego et al. 2008; Alvarez et al. 2009; Oliveira et al. 2012).

Besides micropropagation by stem cuttings, few studies also addressed both direct organogenesis in *Pinus* - including the reports for *P. nigra* (López et al. 1996), *P. pinaster* (Calixto & Pais 1997; Alvarez et al. 2009), *P. ayacahuite* (Saborio et al. 1997), and *P. pinea* (Alonso et al. 2006; Cuesta et al. 2008), and indirect organogenesis, this last one only in *P. elliottii* (Tang et al. 2006). Most of these organogenic studies used mature zygotic embryos or cotyledons of different ages from mature seeds. Tang et al. (2006) reported a successful protocol for indirect organogenesis of *Pinus elliotti* var. *eliiottii* which use 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA) and 2-isopentenyladenine (2iP) for callus induction, and indole-3-butyric acid (IBA), BAP and thidiazuron (TDZ) for shoot differentiation.

After the induction stage, shoot elongation often takes place in hormone-free media and several protocols include the addition of activated charcoal in order to adsorb the hormones previously added (López et al. 1996; Calixto & Pais 1997; Saborio et al. 1997; Azevedo et al. 2001; Tereso et al. 2006; Kalia et al. 2007; De Diego et al. 2008; Cortizo et al. 2009; De Diego et al. 2010).

For the rooting stage of *Pinus* the most used auxins are IBA and NAA, however it has been reported that shoots of *Pinus pinaster* maintained (without subculturing) on elongation medium for 5-6 months formed roots (Calixto & Pais 1997). This "spontaneous" rooting ability was interpreted as being due to a reduction of the medium nutrients, and since then many authors use media with half or less nutrients at this stage then in the previous (e.g. Kalia et al. 2007; Oliveira et al. 2012). The combination of an auxin with a cytokinin, mainly BAP, is commonly used to promote rooting. It has been reported that the presence

of a cytokinin in the rooting medium can prevent apical necrosis (Oliveira et al. 2012). If the induction stage requires long periods of exposure to growth regulators, then it may be advisable to perform root elongation on hormone-free media, frequently with activated charcoal both to prevent browning (e.g. Abdullah et al. 1987), adsorb excessive growth regulators (Saborio et al. 1997; Azevedo et al. 2001; Pinto et al. 2008; Thomas 2008) and to stimulate morphogenesis/rooting (Dumas & Monteuuis 1995; Thomas 2008). The permanence in the dark at some point of the root induction is also described as a positive factor to the rooting process (Calixto & Pais 1997). Overall, it is accepted that a good quality rooting process is essential to the success of the acclimatization phase and plant establishment in the field (Calixto & Pais 1997; Conde et al. 2004).

Despite of several micropropagation protocols described for other *Pinus* species, to our knowledge, reliable protocols for mass micropropagation – from explant to acclimatization – of slash pine remain without scientific demonstration. Our objective was to develop an efficient protocol for rapid multiplication of *Pinus elliotti* var. *eliiotti* for possible incorporation in breeding programs in the near future.

Materials and Methods

Plant Material

Mature seeds obtained from a clonal seed orchard of *Pinus elliotti* var *eliiotti* (PE), were provided by the company Resisul Fortaleza, Lda (São Paulo, Brazil). Seeds were collected in clonal seed orchards between 2007 and 2009 and conserved at 4ºC until utilization.

Seed disinfection and germination assays

For *in vitro* seed establishment and germination, three disinfection protocols (D1, D2, D3) were tested. In all disinfection protocols the seeds were first washed in tap water, then with water with few drops of detergent before disinfection for three minutes in 70% ethanol. Then in protocols D1 and D2, seeds were surface sterilized for 5 minutes in 50% commercial bleach (NaOCl < 5%) followed by three rinses in sterile water and overnight stand in the last water. Then, seeds were surface sterilized in 50% commercial bleach (NaOCl < 5%) for 15 minutes (in D1) and for 20 minutes (in D2). For both protocols (D1,

D2) the seeds were rinsed for three times in sterile water. In protocol D3, after the ethanol disinfection, the seeds were surface sterilized for 15 minutes in 50% commercial bleach (NaOCl < 5%), followed by three rinses in sterile water. Seed coats were removed under sterile conditions in a laminar flow hood, and the megagametophytes inoculated in the germination medium.

Two different germination media (G1 and G2) were tested for evaluation of the effect of sucrose presence in the medium. As basal medium, ¼ strength MS medium (Murashige & Skoog, 1962) was used supplemented with 8 $g.L^{-1}$ agar with no sucrose (G1 medium), or with 30 $g.L^{-1}$ sucrose (G2 medium). The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min.

All the conditions were tested in at least in 32 seeds. Megagametophytes were incubated in a growth chamber at 22 ± 2 °C, for a 16/8-h (day/night) photoperiod, under a photosynthetic photon flux density (PPFD) of approx. 50 ± 10 μmol m^{-2} s^{-1}. The percentages of contamination and germination were analyzed.

Bud induction

For all the experiments, explant shoot apices with 1.5 – 2.0 cm in length excised from 4-weeks old seedlings obtained form optimized germnation protocol were used. For each condition at least 3 independent replicates (with 3 to 4 explants per replicate) were incubated in a growth chamber at 22 ± 2 °C, for a 16/8-h (day/night) photoperiod, under a PPFD of approx. 50 ± 10 μmol m^{-2} s^{-1}. The effects of both phytoregulators concentration, period of exposure and basal media, were analyzed in separate as described below.

Effect of BAP concentration: In this assay the effect of different concentrations of the cytokinin BAP was tested in bud induction. The explants were inoculated for four weeks on five different induction media I1-I5 (Table 1) which have MS for basal medium and concentrations of BAP between 1 and 25 μM.

Effect of induction time: For this assay the medium I5 (Table 1) was used to test different exposure times to the phytoregulator: T1: one week; T2: two weeks; T3: three weeks and T4: four weeks.

Effect of basal medium: In this assay three different basal media, MS, GD (Gresshoff & Doy 1972) and WV5 (Coke 1996) were tested with the same concentration of BAP [I5, I6

and I7 (Table 1)] during four weeks of induction. For the basal medium WV5, two concentrations of BAP [I7 and I8 (Table 1)] were tested during four weeks of induction.

In all the experiments the induction rate (%), the number of shoots obtained after elongation, the average length of the shoots and the explants or shoots senescence after the cycle of multiplication were calculated. Senescence was evaluated with a semiquantitative scale between 0 and 7 where 0 represents a shoot or explant without senescence signals, and 7 represents the greatest degree of senescence that leads to death of the shoot or explant. Results were obtained from one cycle of multiplication (one cycle of multiplication = induction + elongation).

Table 1 – Composition of the culture media used for first cycle bud induction assays.

Designation	Basal	Vitamins	BAP (μM)	Sucrose ($g.L^{-1}$)	Agar ($g.L^{-1}$)
I1	MS	vitMS	0	30	7
I2	MS	vitMS	1	30	7
I3	MS	vitMS	5	30	7
I4	MS	vitMS	10	30	7
I5	MS	vitMS	25	30	7
I6	GD	vitGD	25	30	7
I7	WV5	vitWV5	25	30	7
I8	WV5	vitWV5	10	30	7

* The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min.

Shoot elongation

After induction the explants were transferred to the elongation medium for six weeks, keeping the same environmental conditions used for induction. Three elongation media were used as described in Table 2. The culture medium was selected in order to maintain the same basal medium used in the induction phase, but without growth regulators and with the addition of activated charcoal (AC).

Table 2 - Composition of the elongation culture media used in these work.

Designation	Basal	Vitamins	Sucrose (g. L^{-1})	Agar ($g.L^{-1}$)	AC ($g.L^{-1}$)
A1	MS	vitMS	30	7	2
A2	GD	vitGD	30	7	2
A3	WV5	vitWV5	30	7	2

* The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min.

Rooting assays

The shoots produced in the basal medium WV5 from the assays described above with 1.5 to 3 cm of length were used for rooting assays. In assays of continuous exposure to growth regulators, different concentrations and combinations of IBA (1-2 $mg.L^{-1}$) and NAA (0.2-0.4 $mg.L^{-1}$) were tested (Table 3). In assays of hormonal-shock, a sterile IBA solution (1 $g.L^{-1}$) was applied for different times (between 2.5 and 10 minutes), prior incubation on the culture on medium R5.

For each rooting condition at least 3 replicates (6 to 10 explants per replica) were made. Plant material was incubated in a growth chamber at 22 ± 2 °C, for a 16/8-h (day/night) photoperiod under a photosynthetic photon flux density (PPFD) of approximatly 50 ± 10 μmol $m^{-2}s^{-1}$, during 6 to 8 weeks. In these assays the rooting percentage (%), the number of roots obtained and the largest root length were determined.

Table 3 – Conditions used for rooting assays

Designation	Shock IBA 1 $g.L^{-1}$	Basal	Vitamins	NAA ($mg.L^{-1}$)	IBA ($mg.L^{-1}$)	Sucrose ($g.L^{-1}$)	Agar ($g.L^{-1}$)
R3		½ WV5	½ vitWV5	0.5	---	15	7
R5	10 min 5 min 2.5 min	½ WV5	½ vitWV5	---	---	15	7
R6		½ WV5	½ vitWV5	---	1	15	7
R7		½ WV5	½ vitWV5	---	2	15	7
R8		½ WV5	½ vitWV5	0.2	1	15	7
R9		½ WV5	½ vitWV5	0.4	1	15	7

* The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min.

Acclimatization

In vitro plantlets with roots (>2 mm long) were transferred to pots with a peat-perlite (1:2) mixture and grown in a growth chambre under high relative humidity (RH=95%). This was gradually reduced and after four weeks it reached ~35%. During this stage, plants were fertilized with 1/10 MS basal salt mixture and vaporized with fungicide solutions (solutions of 1 $g.L^{-1}$ benlate®, Rhône-Poulenc) and of 2.5 $g.L^{-1}$ derosal® (Bayer) were alternatively applied once a week. After four weeks, plants survival percentage was evaluated.

True-to-typeness assessment by flow cytometry

Samples from *in vitro* micropropagated plants and from zygotic plants were prepared to obtain nuclear suspensions. For that, small portions (~50 mg) of needles were chopped in Woody Plant Buffer (WPB) (0.2 M Tris–HCl, 4 mM $MgCl_2$, 2 mM EDTA Na_2, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7.5) (Loureiro et al. 2007b). Samples were filtered through a 50 μm nylon filter. To the nuclear suspension 50 μg/ml of both propidium iodide (Fluka) and RNase (Sigma) were added, to stain DNA and degrade ds-RNA, respectively. After incubating for 5 min at ~4ºC, samples were analyzed in a Attune® Acoustic Focusing Cytometer (Life Technologies Applied Biosystems, Vic, Australia) with an air-cooled argon-ion laser operating at 488 nm. Fluorescence was collected and mono- and bi-parametric histograms acquired and analyzed (e.g. Loureiro et al. 2006). Putative contamination with cytosolic compounds was screened and the elimination of contamination by nuclei doublets, partial nuclei, nuclei with associated cytoplasm and other debris was performed as described previously (Loureiro et al. 2006). Around 5,000 nuclei were analyzed per sample. The ploidy levels (and putative occurrence of aneuploidy or polyploidy) were determined by analyzing the G_0/ G_1 peaks position and/or appearance of new G_0/G_1 peaks.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA) and when necessary data were transformed to achieve normality and equality of variance. When these criteria were not satisfied even with transformed data, the non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks was performed. The post hoc analysis was evaluated by

Holm-Sidak or Dunn's Multiple Comparison Test. The significance level was 0.05. All statistical analysis was performed using SigmaPlot for Windows. version 11.0.

Results

Disinfection and Germination assays

The contamination and germination rates (%) achieved with the three protocols (D1-D3), and with the presence/absence of sucrose (G1 and G2) on the contamination and germination rates of slash pine seeds are presented in Figure 1. Despite a trend for lower levels of contamination in D3, these values were not significantly different ($p>0.05$) from the other conditions (D1 and D2). Also, the addition of sucrose to the medium did not positively influence ($p>0.05$) the germination rate compared with the medium without sucrose (Figure 1). Overall, the rates of contamination were consistently lower than 42%, while the maximum rates of germination were reached on G1:D3 (68.75%) and always it was concluded that all protocols tested provided a moderate-high level of germinants that may be consistent for large scale protocols, and the protocol that was adopted for future experiments in this work was the combination of G1:D3.

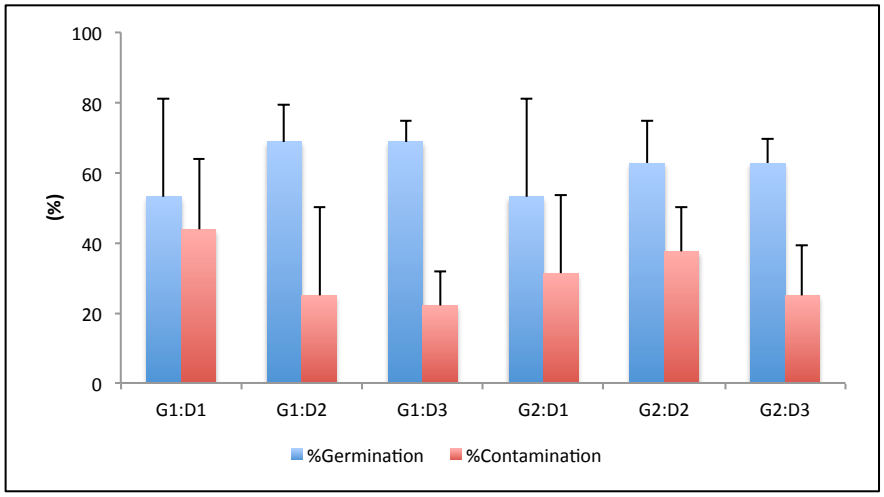

Figure 1 –Effect of different disinfection protocols (D1-D3) and presence of sucrose (G1 and G2) on the contamination and germination rates of PE seeds. Data recorded after four weeks of culture. Means ± SE. $n \geq 32$. Data were analyzed by a one-way analysis of variance (ANOVA) and no significant differences were found.

Bud induction and elongation

The first assay tested the effect of BAP concentration (0 and 25 µM) on the bud induction rate. Results show that the best induction percentage and higher number of shoots per explant were obtained with the highest BAP concentration (25 µM), reaching values of 90% and 5.2, respectively (Figure 2). Differences on the bud induction and growth, as result of the concentration of BAP, were visible after four weeks. Overall, buds formed on higher BAP concentrations were more developed (Figure 3). The number of shoots per explant in response to BAP concentration increased similarly to the induction rate. Lower values (~2 new shoots/explant) were found for the culture medium I1, without BAP, and for I2 with the lowest BAP concentration (1 µM), increasing significantly for concentrations higher than 5 µM, and reaching maximum values for I5 (~6 new shoots/explant). However, this increase of new shoots formation occurred at the expenses of a decrease of their length.

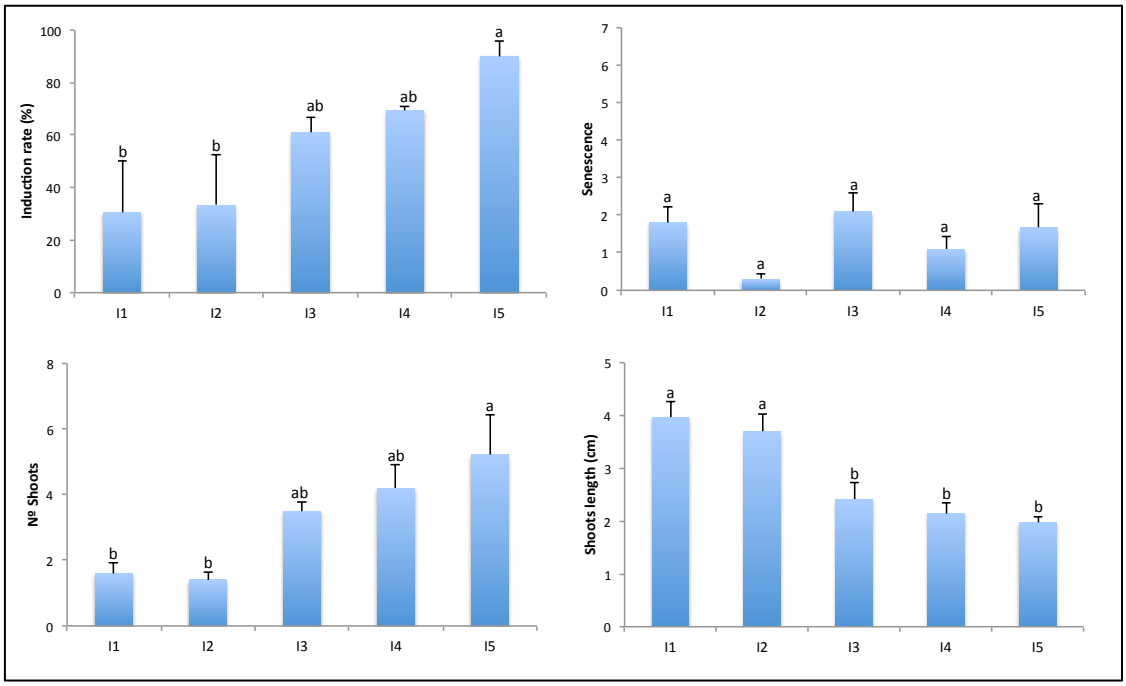

Figure 2 - Effect of BAP concentration on the bud induction percentage, senescence, number and average length of shoots obtained per explant of PE. Data recorded after four weeks of culture in induction medium plus six weeks in elongation medium (A1). Means ± SE. *n* ≥10. I1 (0 µM BAP, A1), I2 (1 µM BAP, A1), I3 (5 µM BAP, A1), I4 (10 µM BAP, A1), I5 (25 µM BAP, A1) – for other media conditions see Table 1. Values marked with different letters have significant differences ($p \leq 0.05$).

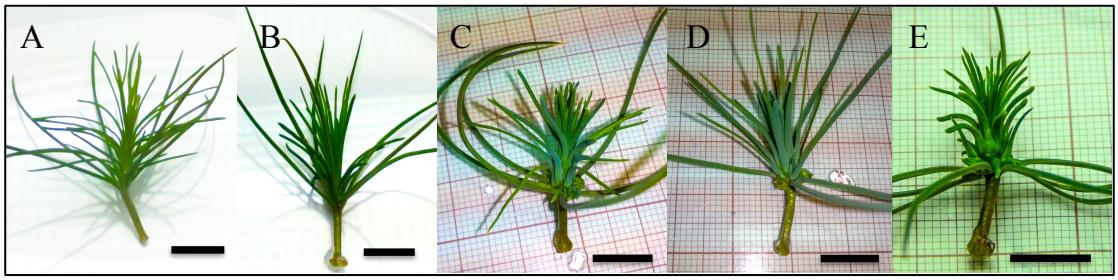

Figure 3 – *In vitro* bud induction of PE for four weeks**.** A – Explant induced in I1; B – Explant induced in I2; C – Explant induced in I3; D - Explant induced in I4; E - Explant induced in I5. Bar=1 cm.

The medium I5 was used to test the effect of different exposure periods (1, 2, 3 or 4 weeks) to the growth regulator. Results show that there are no significant differences ($p>0.05$) in any of the parameters studied (Figure 4). However, overall a higher number of shoots formed per explant was obtained after 4 weeks of exposure, and also after this period, these buds were more evident (Figure 5) than when shorter periods were used.

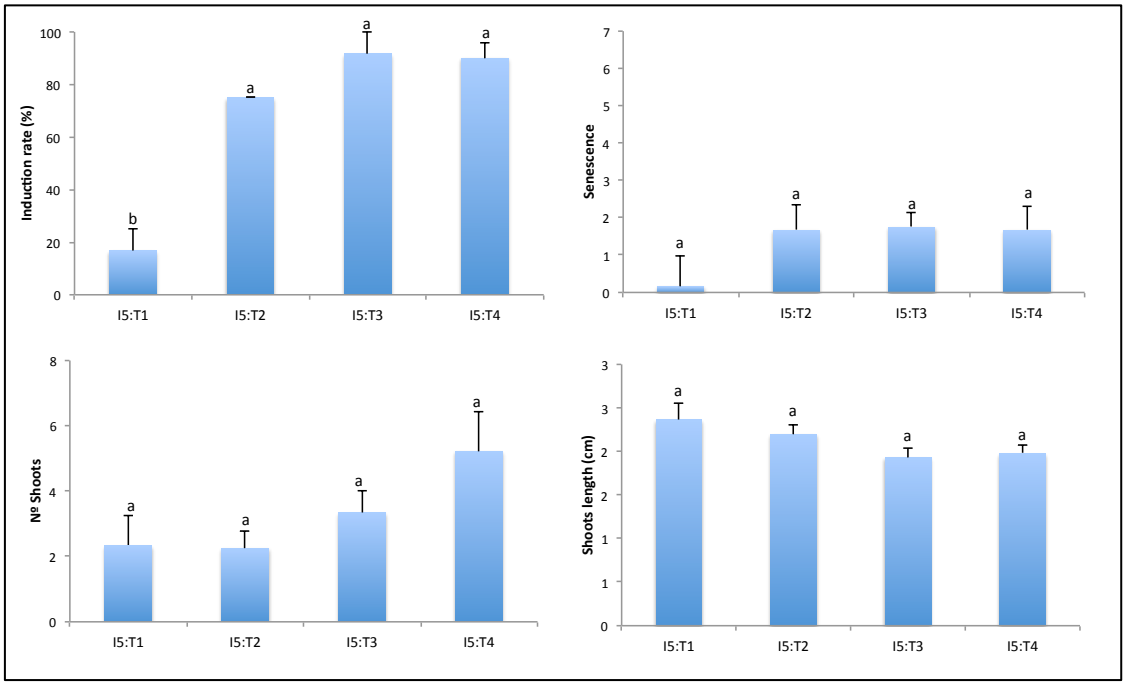

Figure 4 - Effect of induction time on the bud induction percentage, senescence, number and average length of shoots obtained per explant of PE. Data recorded after the refered weeks of culture in induction medium plus six weeks in elongation medium (A1). Means ± SE. $n \geq 10$. T1: one week; T2: two weeks; T3: three weeks and T4: four weeks – for other media conditions see Table 1. Values marked with different letters have significant differences ($p \leq 0.05$).

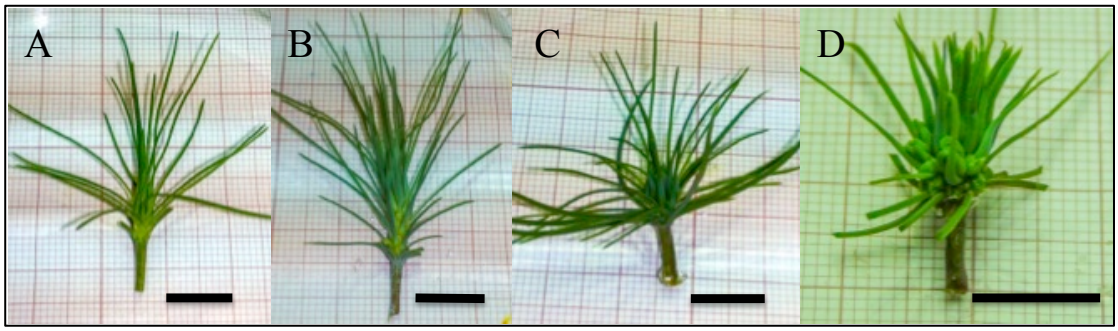

Figure 5 - *In vitro* bud induction of PE in I5. A – Explant induced for 1 week; B – Explant induced for 2 weeks; C – Explant induced for 3 weeks; D - Explant induced for 4 weeks. Bar=1 cm.

Results from the basal medium assay are shown in Figure 6. The three tested basal media led to similar induction rates ($p>0.05$) between 89% and 94%. However, the number of new buds formed in GD was significantly lower ($p<0.05$) than the other two media (1 new shoot/explant *versus* ~6 and 7 per explant for MS and WV5, respectively). WV5 induced less senescence than the other media and newly formed shoots on this medium were significantly longer (3.2 cm long) than shoots formed on MS and GD. So WV5 medium stand out as the more appropriate basal medium to slash pine bud induction.

The comparison between the medium I7 (25 μM of BAP) and I8 with a lower concentration of BAP (10 μM of BAP) show no significant differences ($p>0.05$) for the rate of induction, newly formed shoots per explant and low senescence. However, greater elongation of the new shoots was observed when the induction was made in the medium I8 (~5 cm long), supporting that I8 is better than I7 (Table 4).

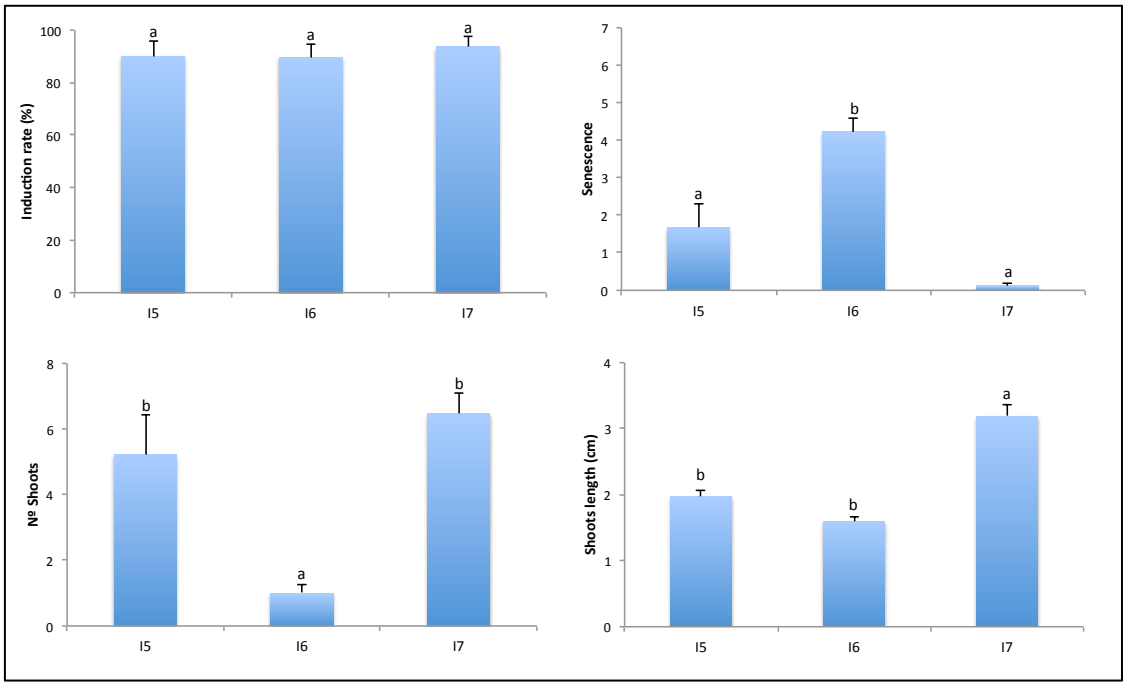

Figure 6 - Effect of basal medium on the bud induction percentage**,** senescence**,** number and average length of shoots obtained per explant of PE. Data recorded after four weeks of culture on MS, GD or WV5 plus six weeks on the respective elongation medium (A1,A2 or A3). Means ± SE. $n \geq 10$. I5 (MS, A1), I6 (GD, A2), I7 (WV5, A3) – for other media conditions see Table 1. Values marked with different letters have significant differences ($p \leq 0.05$).

Table 4 - Effect of BAP concentration on the bud induction percentage, senescence, number and average length of shoots obtained per explant of PE induced in WV5. Data recorded after four weeks of culture in induction medium plus six weeks in elongation medium (A3). Means ± SE. $n \geq 10$. Data were analyzed by a one-way analysis of variance (ANOVA) and no significant differences were found.

Condition	Induction rate (%)	Senescence	NºShoots	Shoots length (cm)
I7	93.75±4.09	0.13±0.06	6.47±0.63	3.19±0.17
I8	92.86±4.61	0.55±0.20	6.55±0.77	5.02±0.27

Rooting and Acclimatization

Shoots with more than 1.5 cm long, obtained on I7 and I8, were used for the rooting studies. For this, the main basal medium (WV5) was maintained, but with half concentration of the nutrients (1/2 WV5).

The auxin variable (alone or combinations) was assessed on rooting ability (Table 5). The results obtained show that the formation of adventitious roots is more efficient in the rooting media that include only NAA (Table 5). Maximum rooting percentages were

achieved on R3 (84.44%), however, also higher rates of callus were observed on these plants (Figure 7A). This fact raised the question if normally functional rhizogenesis has occurred. Other combinations, IBA-containing media, despite leading to lower rooting efficiency did not induce callus formation (Figure 7B and C).

All plants obtained were acclimatized. Curiously, plants obtained on NAA-media had lower acclimatization success than plants obtained on an IBA rich-medium or with the two auxins combined.

Regarding hormone shock (R5), this strategy led to heterogenous responses, although better results were achieved with a shock of 5 min IBA 1g/L, leading to ~45% rooting and 30% of acclimatization. Higher rates were achieved with R7, R8 and R9 conditions, with rooting success of almost 44%, 60% and 47%, respectively, and acclimatization rates of around 89%, 45% and 57% respectively.

Table 5 – Effect of different rooting treatments on the number of roots, length of largest root, rooting and acclimatization percentage of shoots of PE. Data recorded after four six to eight weeks in rooting medium. Means ± SE. $n \geq 20$. Values marked with different letters have significant differences ($p \leq 0.05$).

Culture Medium	Shock IBA 1 $g.L^{-1}$	Nº roots			Largest root (mm)			% Rooting			% Acclimatization
R5	10 min	1.00	a		7.00	a		3.33 ± 3.33	a		100.00
R5	5 min	1.81 ± 0.19	b		10.16 ± 0.96	a		44.81 ± 11.23	ab		29.41
R5	2.5 min	1.33 ± 0.21	a		10.50 ± 2.17	a		16.67 ± 9.62	b		83.33
R3		5.63 ± 0.47	B		14.40 ± 1.67	A		84.44 ± 3.57	A		15.87
R6		2.00 ± 0.45	A		5.83 ± 0.98	AB		19.39 ± 3.72	B		66.67
R7		1.85 ± 0.42	A		5.15 ± 0.97	AB		43.33 ± 12.02	BC		88.89
R8		3.33 ± 0.59	A		8.80 ± 1.60	AB		60.00 ± 5.77	AC		44.44
R9		6.71 ± 1.65	A		4.07 ± 0.68	B		46.67 ± 6.67	BC		57.14

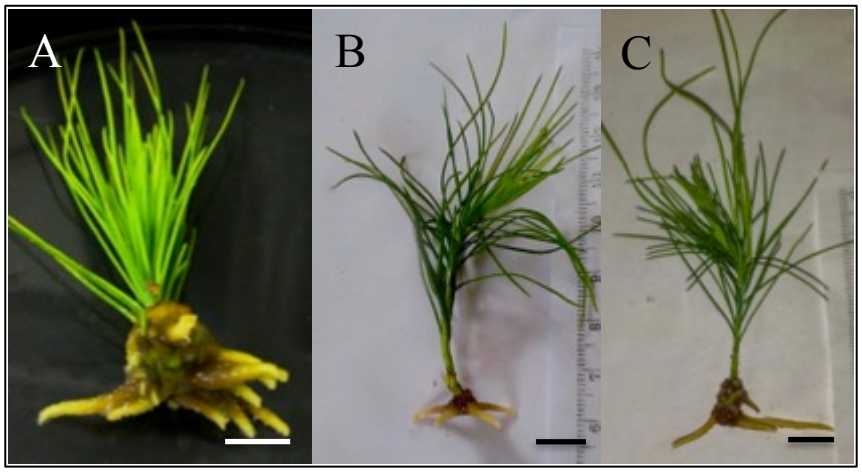

Figure 7 – PE rooted shoots. A – Rooting promoted in R3; B – Rooting promoted in R7; C – Rooting promoted in R8. Bar=1 cm.

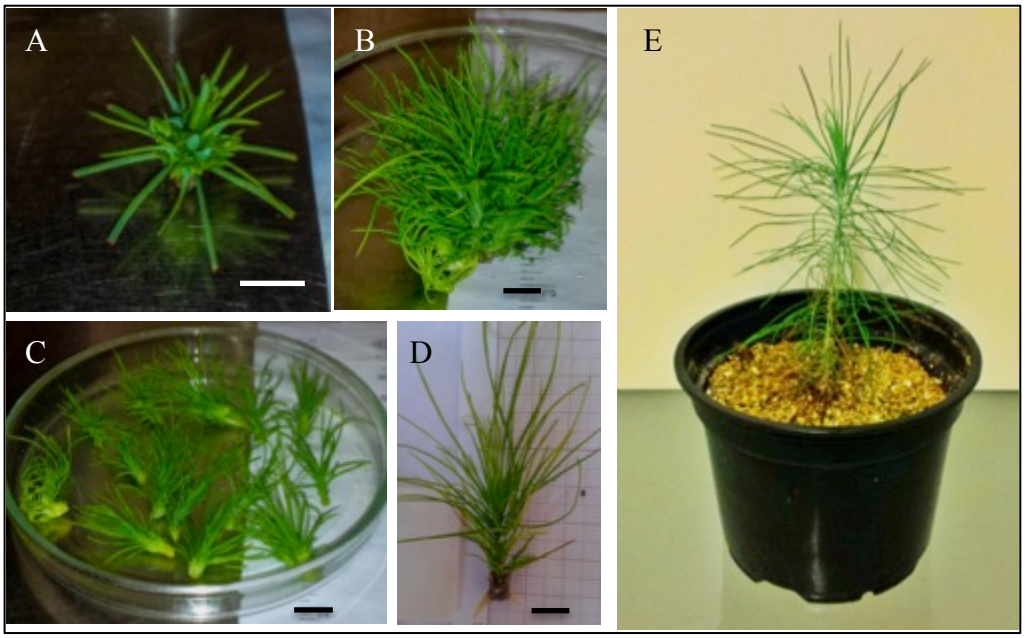

Figure 8 - Micropropagation protocol. A – Explant after bud induction in I8 for four weeks; B - Explant after elongation in A3 for six weeks; C – Shoots separated; D – Rooted shoot after six weeks in R7; E – Plant produced with the micropropagation protocol established. Bar=1 cm.

Genetic stability analysis

The ploidy level of micropropagated plants and of zygotic-derived plants was analyzed by flow cytometry (FCM), using leaves as nuclei source. FCM histograms showed typical diploid profiles (Figure 9). G1 peaks had a mean CV value of 4.31 for zygotic seedlings, and of 5.15 for micropropagated plants. All analyzed nuclei, of both populations, had

similar DNA-ploidy profiles independently of the origin of the plants (zygotic vs. micropropagated).

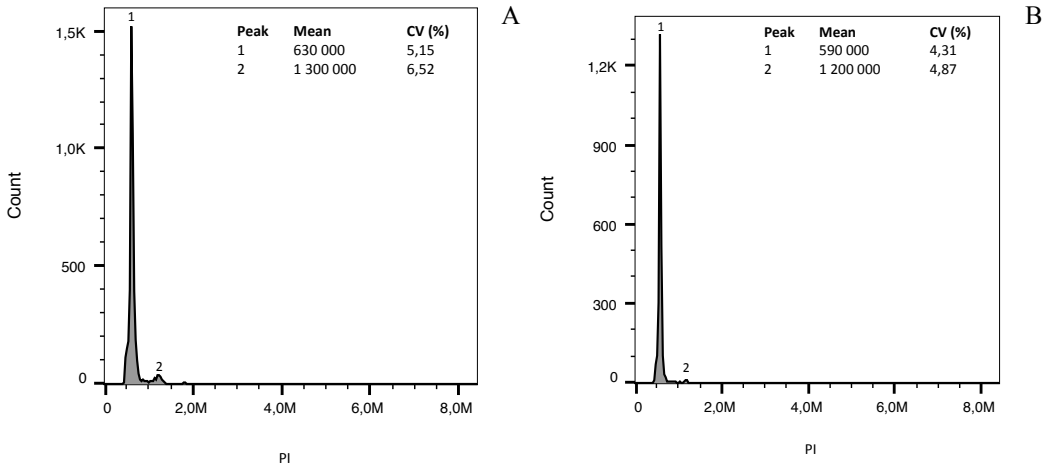

Figure 9 - Histograms of relative fluorescence intensity (PI) obtained for *Pinus elliottii* nuclei: A) from micropropagated plant leaves; B) from seedling plant leaves. Peak 1 – G0/G1 ; Peak 2 – G2; CV – coefficient of variation.

Discussion

In this work mature seeds obtained from a clonal seed orchard of *Pinus elliotti* var. *eliiotti* were used to establish a reproducible mass micropropagation protocol for this species, including all stages from disinfection to plant acclimatization.

Seed disinfection strategies and germination success are intimately associated. Our data demonstrated that germination was not improved by disinfection strategies including an over nightstand in water or the inclusion of sucrose on the culture medium, supporting the selection of the protocol G1:D3. D1 and D2 approaches favoured a long period for seed imbibition (the tissue rehydration occurs, stimulating metabolic activities that result in the supply of energy and nutrients necessary for the resumption of the embryonic axis growth), but a short period of seed incubation provides the necessary level of moisture, which combined with seed coat removal prior megagametophytes inoculation provided the best results in this species. At the large-scale level, this protocol also benefits the operational level as it is not time-consuming and may provide a consistent number of new axenic seedlings germinated/week (>53% of inoculated seeds). The excess moisture may cause a

decrease in germination because it prevents the penetration of oxygen and reduces all the resulting metabolic process (Floriano 2004a).

The present study demonstrated that new buds can be induced from seedling explants of *P. elliottii*, which is in accordance with the results obtained for the same species by Burns and co-workers (1991) although the full protocol was not provided. Similar explants have been successfully cultured in other pine species, like *P. sylvestris, P. pinaster, P. roxburghii* and *P. massoniana* (Žel et al. 1988; Azevedo et al. 2001; Tereso et al. 2006; Kalia et al. 2007; Zhu et al. 2010).

For industrial use, it is required to optimize conditions of bud induction in this species. The starting conditions (BAP concentrations and basal media) tested here were selected from literature on *Pinus* micropropagation. Overall, several pine micropropagation protocols use the basal medium MS (Žel et al. 1988; López et al. 1996; Kalia et al. 2007). Also, most of those works use BAP ranging from 1-50 μM in the induction of axillary shoots, independently of the explant source (Žel et al. 1988; Burns et al. 1991; Kalia et al. 2007; De Diego et al. 2008; Oliveira et al. 2012), and our best results (10 - 25 μM) support that *P. elliottii* has a behaviour similar to other *Pinu*s species. They are particularly close to the BAP concentrations (10- 20 μM) required by *P. roxburghii* (Kalia et al. 2007). Our results also demonstrate that the presence of a cytokinin alone is sufficient to induce bud formation in slash pine, similarly to other pine species as *P. pinaster, P. taeda, P. pinea, P. roxburghii* and *P. ayacahuite* (Calixto & Pais 1997; Saborio et al. 1997; Alonso et al. 2006; Kalia et al. 2007; Alvarez et al. 2009; Oliveira et al. 2012). On the other hand, the fact that new buds formed on media containing BAP concentrations above 5 μM had a compromised elongation is consistent with the results described for *P. sylvestris,* as described by Zel et al. (1988).

It is well known that *in vitro* conditions can affect the plants genetic stability, leading to somaclonal variation (e.g. Leal et al. 2006; Lopes et al. 2006; Loureiro et al. 2007a). Since the frequency of variation increases with the time in culture another important factor to optimize in the bud induction is the period of exposure to the growth regulator, which was found to reach higher values after 4 weeks of exposure to BAP. It has been reported for other pines, for the same type of explants, that the length of exposure to the growth regulators may vary between 3 to 4 weeks for bud induction (Azevedo et al. 2001;

Nandwani et al. 2001; Tereso et al. 2006; Kalia et al. 2007; Zhu et al. 2010) which complies with our results.

The basal medium is another importante factor that can influence the bud induction and shoot elongation (e.g. Azevedo et al. 2001; Zhang et al. 2003; Sul & Korban 2004; De Diego et al. 2008; De Diego, Montalbán & Moncaleán 2010; Oliveira et al. 2012). As mentioned above, many authors have described successful protocols for micropropagation using MS as basal medium but, in comparative studies, better results have been found with other formulations (e.g. Burns et al. 1991; Lv & Huang 2012; Oliveira et al. 2012). Considering the lack of available protocols for mass micropropagation of slash pine, the three basal media used here are among the basal most widely used in this genus. Better results were achieved with WV5 followed by MS and finally GD. Curiously, our results are not in accordance with Burns and co-workers (1991) who reported for slash pine that GD provided better sprouting than MS.

Our results are more in line with those reported for *P. taeda* (Coke 1996; Oliveira et al. 2012). In particular, Oliveira et al (2012) compared MS and WV5, and showed that the last medium provided better sprouting and elongation. Also, Coke (1996) reported that the balance of salts in WV5 medium favors an optimal development of *in vitro* cultures of *P. taeda*. These culture media diverge on the nitrogen availability, where WV5 has lower amount of nitrogen than GD and MS culture media. Oliveira et al. (2012) had referred that high concentrations of N in culture medium formulations may have a toxic effect in some species, which may be related to the higher senescence values obtained for MS and GD in this work.

Other strategies such as shoot decapitation (Parasharami et al. 2003; Kalia et al. 2007), were proposed to release apical dominance and faster shoot elongation. Compared with not decapitated shoots, this procedure, however, revealed no benefits in slash pine shoots (data not shown).

Once tissues exposure to high amounts (period and/or concentration) of growth regulators may induce somaclonal variance, we succeeded to demonstrate that using WV5 and lowering BAP concentrations (10 μM) allowed excellent results for shoots elongation. In fact, among all tested media (I1-I8), both I7 and I8 were by far the best media particularly

by combining induction of low senescence and higher shoot length being currently recommended as best media for *P. elliottii* multiplication stage.

Pine shoot rooting is usually consired necessary, despite few reports on spontaneously rooting after long periods on elongation medium. It was the case reported by Calixto and Pais (1997) to *P. pinaster*, where 50% of spontaneous rooting was achieved. In our work spontaneous rooting only occurred ocasionally, supporting that rooting treatment is essential to complete the micropropagation protocol for this species.

In all rooting conditions tested WV5 nutrients and sucrose concentrations were reduced to half in order to facilitate root formation.

It is largely consensual for several species that reducing nutrients availability may promote rhizogenesis (Calixto & Pais 1997; Kalia et al. 2007). IBA and the synthetic auxin NAA are commonly used to induce root meristem differentiation and for several pines a combination of the two auxins may benefit root induction (López et al. 1996; Montalban et al. 2011). These auxins are also used separately for long-term exposure or for hormone-shock procedures. For example, Calisto and Pais (1997) used a shock of IBA solution (396.6 μM for 24h) to induce rooting on shoots of *P. pinaster,* with rooting success rates of ~60%. However, most published rooting protocols for other *Pinus* species used long-term exposure NAA (e.g, ranging from 0.2 to ~ 2 $mg.L^{-1}$) with efficiency percentages ranging from 60 to 90% (Azevedo et al. 2001; Alonso et al. 2006; Kalia et al. 2007; De Diego et al. 2008; Alvarez et al. 2009).

For *P. elliottii* best rooting results (nearly 85%) were obtained by prolonged exposure to 0.5 $mg.L^{-1}$ NAA. These rooting values are considerably high and already suitable for a mass production program. It overcomes most results obtained with the same auxin to other pines as *P. kesiya* (67%) (Nandwani et al. 2001), *P. pinaster* (57%) (De Diego et al. 2008), *P. massoiana* (82%) (Zhu et al. 2010), *P. roxburghii* (60%) (Kalia et al. 2007) and *P. caribaea* (57%) (Halos & Go 1993). Other combinations also originated roots though at lower rates (47% to 60% on media with IBA and NAA combinations, and 19% to 43% for the long exposure just to IBA), but callus formation was absent or residual. Yet, NAA alone induced excessive basal *callus*, which may promote dysfunctional roots (compromising plants viability). Shoot-root function inneficacy was demonstrated by the low acclimatization rate of plantlets formed with this protocol (16%). Contrarily, plants

formed on IBA-rich media (with no callus formation) had higher surviving rates (44 to 89%), supporting higher root functionality, crucial to the acclimatization process.

Acclimatization data support that for mass cloning of *P. elliottii*, the use of IBA-containing media may be recommended for rooting stages. Our results are similar to the ones reported by Montalbán et al. (2011) for *P. radiata*, in which better rooting percentages were achieved with NAA > NAA+IBA > IBA. However, the performance of those *in vitro* plants was not evaluated during acclimatization. Our rooting protocol supports a large mass propagation program, as using the R7 medium we can reach a rentability of ~40 acclimatized plants/100 initial shoots on rooting medium.

Genetic fidelity is a major issue in any micropropagation protocol. FCM is currently the preferred and more accurate method of genome size determination (e.g. Hall 2000; Prado et al. 2010). Alone or combined with other genetic/molecular markers, DNA-ploidy has been largely used to assess genetic stability in micropropagation protocols of forest species (e.g. López et al. 1996; Conde et al. 2004; Fernandes et al. 2008; Marum et al. 2009; Brito et al. 2010). However it has only occasionaly been used on *Pinus* species and once to assess genetic fidelity in *P. pinaster* (Marum et al. 2009). FCM allowed a better knowledge of the genome of several *Pinus* species (*P. heldreichii*, *P. peuce*, *P. nigra*, *P. sylvestris* and *P. mugo* and *P. uncinata)* with, for example, discrimination of subpopulations (Bogunic et al. 2003; 2011), and the evolutionary profiles of it, and by Hall et al. (2000) to determine intraspecicies variation in pine DNA content in tropical pines subgenus *Pinus* and the subgenus *Strobus*. Also, hybrids of *P. elliottii* Engelm x *P. caribaea* var. *hondurensis* Morelet and of *P. wallichiana* A.B. Jacks. x *P. strobus* L. (*P. x schwerinii*) and *P. lambertiana* Dougl. x *P. armandii* Franchlet were analyzed for their DNA stability by flow cytometry (Williams et al. 2002). In the case of *P. elliottii*, CV values of the G1 peaks are within the recomended CV values for woody species (e.g. Loureiro et al. 2006; Leal et al. 2006; Brito et al. 2010). The fact that micropropagated plants had DNA-ploidy similar to those of zygotic ones means that no changes in ploidy level (e.g. aneuploidy, polyploidy) were detected in the micropropagated plants. So, the reported protocol also ensures true-to-typeness of cloned plants.

Conclusion

The present work demonstrates that a simple and efficient protocol for micropropagation, that uses as explants seedling-shoot apices, can be applied for routine micropropagation of *P. elliottii*, a *Pinus* species with major economic value. We assessed crucial variables in the different stages of the micropropagation process, including: disinfection, induction, elongation, rooting and acclimatization. All stages were optimized in order to achieve a satisfactory routine protocol that may within a short period be used to support large mass cloning (Figure 10).

We conclude that WV5 is the best basal medium, and that there is no need to add auxin for bud induction. Best results were obtained for shoot induction with 10 - 25 μM of BAP. Rooting of the shoots can be achieved with the addition of IBA alone or in combination with NAA, using half-strength WV5 medium. Rooting was a critical stage, but satisfactory data were obtained and plants successfully acclimatized. Finally, we believe that this protocol will represent a turn-over step to slash pine breeding programs all over the world, as besides its efficacy, it was also demonstrated that it does not promote large mutations, and plants showed by FCM true-to-typeness.

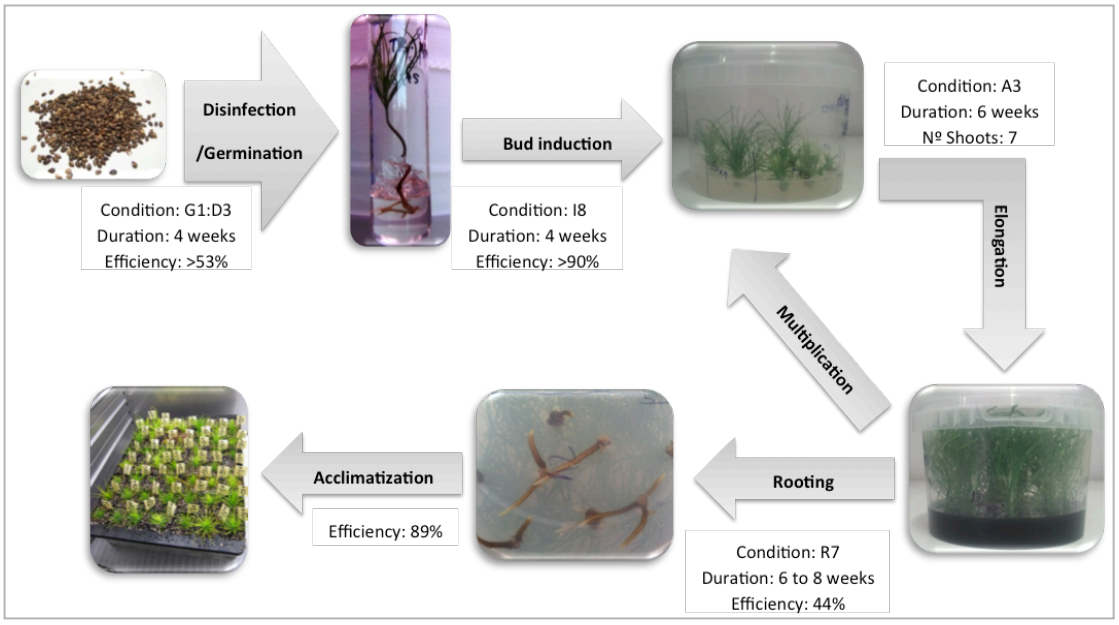

Figure 10 - Optimized protocol for the micropropagation of *Pinus elliottii* var. *elliottii*.

References

Abdullah, A A, M M Yeoman, and J Grace. 1987. "Micropropagation of Mature Calabrian Pine (*Pinus brutia* Ten.) from Fascicular Buds." *Tree Physiology* 3 (2): 123–36.

Alonso, Pablo, Paloma Moncaleán, Belén Fernández, Ana Rodríguez, Maria Luz Centeno, and Ricardo J. Ordás. 2006. "An Improved Micropropagation Protocol for Stone Pine (*Pinus pinea* L.)." *Annals of Forest Science* 63 (8). EDP Sciences: 879–85. doi:10.1051/forest:2006071.

Alvarez, J. M., J. Majada, and R. J. Ordas. 2009. "An Improved Micropropagation Protocol for Maritime Pine (*Pinus pinaster* Ait.) Isolated Cotyledons." *Forestry* 82 (2): 175–84. doi:10.1093/forestry/cpn052.

Azevedo, C, J Maia, C Lopes, and F Gomes. 2001. "Micropropagação Do Pinheiro Bravo (*Pinus pinaster* Aiton)." *Melhoramento* 37: 62–66.

Bogunic, F, E Muratovic, S C Brown, and S Siljak-Yakovlev. 2003. "Genome Size and Base Composition of Five *Pinus* Species from the Balkan Region." *Plant Cell Reports* 22 (1): 59–63. doi:10.1007/s00299-003-0653-2.

Bogunić, Faruk, Sonja Siljak-Yakovlev, Edina Muratović, Fatima Pustahija, and Safer Medjedović. 2011. "Molecular Cytogenetics and Flow Cytometry Reveal Conserved Genome Organization in *Pinus mugo* and *P. uncinata*." *Annals of Forest Science* 68 (1): 179–87. doi:10.1007/s13595-011-0019-9.

Brito, Gina, Tina Lopes, João Loureiro, Eleazar Rodriguez, and Conceição Santos. 2010. "Assessment of Genetic Stability of Two Micropropagated Wild Olive Species Using Flow Cytometry and Microsatellite Markers." *Trees* 24 (4): 723–32. doi:10.1007/s00468-010-0442-9.

Burns, J A, O J Schwarz, and S E Schlarbaum. 1991. "Multiple Shoot Production from Seedling Explants of Slash Pine (*Pinus elliottii*, Engelm.)." *Plant Cell Reports* 10 (9): 439–43. doi:10.1007/BF00233810.

Calixto, Filomena, and M. Salomé Pais. 1997. "Adventitious Shoot Formation and Plant Regeneration from *Pinus pinaster* Sol. Ex Aiton." *In Vitro Cellular & Developmental Biology - Plant* 33 (2): 119–24. doi:10.1007/s11627-997-0008-8.

Campbell, Malcolm M, Amy M Brunner, Helen M Jones, and Steve H Strauss. 2003. "Forestry's Fertile Crescent: The Application of Biotechnology to Forest Trees." *Plant Biotechnology Journal* 1 (3): 141–54.

Coke, J E. 1996. "Basal Nutrient Medium for in Vitro Cultures of Loblolly Pines." Google Patents.

Conde, P, J Loureiro, and C Santos. 2004. "Somatic Embryogenesis and Plant Regeneration from Leaves of Ulmus Minor Mill." *Plant Cell Reports* 22 (9): 632–39. doi:10.1007/s00299-003-0735-1.

Cortizo, Millán, Nuria de Diego, Paloma Moncaleán, and Ricardo Javier Ordás. 2009. "Micropropagation of Adult Stone Pine (*Pinus pinea* L.)." *Trees* 23 (4): 835–42. doi:10.1007/s00468-009-0325-0.

Cuesta, Candela, Ricardo J. Ordás, Belén Fernández, and Ana Rodríguez. 2008. "Clonal Micropropagation of Six Selected Half-Sibling Families of *Pinus pinea* and Somaclonal Variation Analysis." *Plant Cell, Tissue and Organ Culture* 95 (1): 125–30.

doi:10.1007/s11240-008-9412-y.

De Diego, N., I. A. Montalbán, E. Fernandez de Larrinoa, and P. Moncaleán. 2008. "In Vitro Regeneration of *Pinus pinaster* Adult Trees." *Canadian Journal of Forest Research* 38 (10): 2607–15. doi:10.1139/X08-102.

De Diego, N., I.A. Montalbán, and P. Moncaleán. 2010. "In Vitro Regeneration of Adult *Pinus sylvestris* L. Trees." *South African Journal of Botany* 76 (1): 158–62. doi:10.1016/j.sajb.2009.09.007.

Dumas, Elisabeth, and Olivier Monteuuis. 1995. "In Vitro Rooting of Micropropagated Shoots from Juvenile and Mature *Pinus pinaster* Explants: Influence of Activated Charcoal." *Plant Cell, Tissue and Organ Culture* 40 (3): 231–35. doi:10.1007/BF00048128.

Fernandes, P., E. Rodriguez, G. Pinto, I. Roldan-Ruiz, M. De Loose, and C. Santos. 2008. "Cryopreservation of Quercus Suber Somatic Embryos by Encapsulation-Dehydration and Evaluation of Genetic Stability." *Tree Physiology* 28 (12): 1841–50. doi:10.1093/treephys/28.12.1841.

Floriano, EP. 2004a. "Germinação E Dormência de Sementes Florestais." Santa Rosa.

Gresshoff, P M, and C H Doy. 1972. "Development and Differentiation of Haploid Lycopersicon Esculentum (tomato)." *Planta* 107 (2): 161–70. doi:10.1007/BF00387721.

Hall, S, W. S. Dvorak, JS Johnston, HJ Price, and CG Williams. 2000. "Flow Cytometric Analysis of DNA Content for Tropical and Temperate New World Pines." *Annals of Botany* 86 (6): 1081–86. doi:10.1006/anbo.2000.1272.

Halos, Saturnina C., and Nancy E. Go. 1993. "Micropropagation of *Pinus caribaea* Morelet." *Plant Cell, Tissue and Organ Culture* 32 (1): 47–53. doi:10.1007/BF00040115.

Kalia, R. K., S. Arya, S. Kalia, and I. D. Arya. 2007. "Plantlet Regeneration from Fascicular Buds of Seedling Shoot Apices of *Pinus roxburghii* Sarg." *Biologia Plantarum* 51 (4): 653–59. doi:10.1007/s10535-007-0138-1.

Leal, F, J Loureiro, E Rodriguez, M S Pais, C Santos, and O Pinto-Carnide. 2006. "Nuclear DNA Content of *Vitis vinifera* Cultivars and Ploidy Level Analyzes of Somatic Embryo-Derived Plants Obtained from Anther Culture." *Plant Cell Reports* 25 (9): 978–85. doi:10.1007/s00299-006-0162-1.

Lopes, T, G Pinto, J Loureiro, A Costa, and C Santos. 2006. "Determination of Genetic Stability in Long-Term Somatic Embryogenic Cultures and Derived Plantlets of Cork Oak Using Microsatellite Markers." *Tree Physiology* 26: 1145–52.

López, Marián, José Pacheco, Roberto Rodríguez, and Ricardo J. Ordás. 1996. "Regeneration of Plants from Isolated Cotyledons of Salgareño Pine (*Pinus nigra* Arn. ssp.Salzmannii (Dunal) Franco)." *In Vitro Cellular & Developmental Biology - Plant* 32 (2): 109–14. doi:10.1007/BF02823140.

Loureiro, J, E Rodriguez, J Dolezel, and C Santos. 2006. "Flow Cytometric and Microscopic Analysis of the Effect of Tannic Acid on Plant Nuclei and Estimation of DNA Content." *Annals of Botany* 98 (3): 515–27. doi:10.1093/aob/mcl140.

Loureiro, J., A. Capelo, G. Brito, E. Rodriguez, S. Silva, G. Pinto, and C. Santos. 2007a. "Micropropagation of Juniperus Phoenicea from Adult Plant Explants and Analysis of Ploidy Stability Using Flow Cytometry." *Biologia Plantarum* 51 (1): 7–14. doi:10.1007/s10535-007-0003-2.

Loureiro, João, Eleazar Rodriguez, Jaroslav Dolizel, and Conceição Santos. 2007b. "Two New

Nuclear Isolation Buffers for Plant DNA Flow Cytometry: A Test with 37 Species." *Annals of Botany* 100: 875–888. doi:10.1093/annbot/mcm152.

Lv, Chengqun, and Baoling Huang. 2012. "Stem Tissue Culture of *Pinus elliottii* × *Pinus caribaea*." In *2012 International Conference on Biomedical Engineering and Biotechnology*, 1700–1703. IEEE. doi:10.1109/iCBEB.2012.366.

Marum, Liliana, João Loureiro, Eleazar Rodriguez, Conceição Santos, M. Margarida Oliveira, and Célia Miguel. 2009. "Flow Cytometric and Morphological Analyzes of *Pinus pinaster* Somatic Embryogenesis." *Journal of Biotechnology* 143 (4): 288–95. doi:10.1016/j.jbiotec.2009.08.001.

Montalban, I. A., N. De Diego, and P. Moncalean. 2011. "Testing Novel Cytokinins for Improved in Vitro Adventitious Shoots Formation and Subsequent Ex Vitro Performance in *Pinus radiata*." *Forestry* 84 (4): 363–73. doi:10.1093/forestry/cpr022.

Nandwani, D, S Kumaria, and P Tandon. 2001. "Micropropagation of *Pinus kesiya* Royle Ex Gord (Khasi Pine)." *Gartenbauwissenschaft* 66 (2): 68–71.

Oliveira, LF, LLF Ribas, M Quoirin, HS Koehler, E Amano, and AR Higa. 2012. "Micropropagation of *Pinus taeda* L. from Juvenile Material." *Tree and Forestry Science and Biotechnology* 6 (1): 96–101.

Parasharami, VA, IS Poonawala, and RS Nadgauda. 2003. "Bud Break and Plantlet Regeneration in Vitro from Mature Trees of *Pinus roxburghii* Sarg." *CURRENT SCIENCE* 84 (2): 203–8. http://tejas.serc.iisc.ernet.in/~currsci/jan252003/203.pdf.

Pinto, G, Y-S Park, L Neves, C Araújo, and C Santos. 2008. "Genetic Control of Somatic Embryogenesis Induction in *Eucalyptus globulus* Labill." *Plant Cell Reports* 27 (6): 1093–1101. doi:10.1007/s00299-008-0532-y.

Pinto, G, S Silva, Y Park, L Neves, C Araújo, and C Santos. 2008. "Factors Influencing Somatic Embryogenesis Induction in *Eucalyptus globulus* Labill.: Basal Medium and Anti-Browning Agents." *Plant Cell, Tissue and Organ Culture* 95 (1): 79–88. doi:10.1007/s11240-008-9418-5.

Pinto, G, H Valentim, A Costa, S Castro, and C Santos. 2002. "Somatic Embryogenesis in Leaf Callus from a Mature *Quercus suber* L. Tree." *In Vitro Cellular & Developmental Biology - Plant* 38 (6): 569–72. doi:10.1079/IVP2002352.

Prado, María Jesús, Eleazar Rodriguez, Laura Rey, María Victoria González, Conceição Santos, and Manuel Rey. 2010. "Detection of Somaclonal Variants in Somatic Embryogenesis-Regenerated Plants of *Vitis vinifera* by Flow Cytometry and Microsatellite Markers." *Plant Cell, Tissue and Organ Culture (PCTOC)* 103 (1): 49–59. doi:10.1007/s11240-010-9753-1.

Saborio, F., W. S. Dvorak, J. K. Donahue, and T. A. Thorpe. 1997. "In Vitro Regeneration of Plantlets from Mature Embryos of *Pinus ayacahuite*." *Tree Physiology* 17 (12): 787–96. doi:10.1093/treephys/17.12.787.

Sul, III-Whan, and Schuyler S. Korban. 2004. "Effects of Salt Formulations, Carbon Sources, Cytokinins, and Auxin on Shoot Organogenesis from Cotyledons of *Pinus pinea* L." *Plant Growth Regulation* 43 (3): 197–205. doi:10.1023/B:GROW.0000046013.47892.4f.

Tang, Wei, Ronald J Newton, and Thomas M Charles. 2006. "Plant Regeneration through Multiple Adventitious Shoot Differentiation from Callus Cultures of Slash Pine (*Pinus elliottii*)." *Journal of Plant Physiology* 163 (1): 98–101. doi:10.1016/j.jplph.2005.04.030.

Tereso, S, S Gonçalves, L Marum, M Oliveira, J Maroco, and C Miguel. 2006. "Improved Axillary and Adventitious Bud Regeneration from Portuguese Genotypes of *Pinus pinaster* Ait."

Propagation of Ornamental Plants 6 (1): 24–33.

Thomas, T Dennis. 2008. "The Role of Activated Charcoal in Plant Tissue Culture." *Biotechnology Advances* 26 (6): 618–31. doi:10.1016/j.biotechadv.2008.08.003.

Williams, Claire G., Karen L. Joyner, Lisa D. Auckland, Spencer Johnston, and H. James Price. 2002. "Genomic Consequences of Interspecific *Pinus* Spp. Hybridization." *Biological Journal of the Linnean Society* 75 (4): 503–8. doi:10.1046/j.1095-8312.2002.00038.x.

Žel, J., N. Gogala, and M. Camloh. 1988. "Micropropagation of *Pinus sylvestris*." *Plant Cell, Tissue and Organ Culture* 14 (3): 169–75. doi:10.1007/BF00043407.

Zhang, Huaibi, Kathryn Joan Horgan, Paul Hugh Stewart Reynolds, and Paula Elizabeth Jameson. 2003. "Cytokinins and Bud Morphology in *Pinus radiata*." *Physiologia Plantarum* 117 (2): 264–69. doi:10.1034/j.1399-3054.2003.00026.x.

Zhu, Li-Hua, Xiao-Qin Wu, Hong-Ye Qu, Jing Ji, and Jian-ren Ye. 2010. "Micropropagation of *Pinus massoniana* and Mycorrhiza Formation in Vitro." *Plant Cell, Tissue and Organ Culture (PCTOC)* 102 (1): 121–28. doi:10.1007/s11240-010-9711-y.

II.2 GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF *IN VITRO* AND *EX VITRO* SEEDLINGS OF *PINUS ELLIOTTII*: A CONTRIBUTION TO BREEDING PROGRAMS

Chapter prepared to be submitted as an original paper to a SCI journal:

Nunes S, Dias MC, Moutinho-Pereira J, Correia C, Oliveira H, de Oliveira JMPF, Pereira VT, Almeida T, Marum L, Santos C. 2016. Genetic and physiological characterization of *in vitro* and *ex vitro* seedlings of *Pinus elliottii*: a contribution to breeding programs.

Abstract

Pinus elliottii var. *elliottii* is a pine species with enormous economic value particularly for timber and resin industries, and is subjet of high pressure for genetic improvement and cloning elite genotypes. We have recently developed a robust micropropagation protocol for this species. Plants performance needs to be evaluated to validate this protocol for further mass propagation. Micropropagated plants (with six months after acclimatization) and seed-derived plants with the same age were compared regarding photosynthesis (gas-exchange and chlorophyll *a* fluorescence), carbohydrates and pigments content, water status, DNA content and cell cycle dinamics. We demonstrate that the micropropagated plants had overall a physiological performance quite similar to seed-derived plants, suggesting that these micropropagated plants achieved the acclimatization process with success. In particular, except for CO_2 assimilation rate and TSS content, no major differences between micropropagated and seed-derived plants in terms of relative water content, chlorophyll *a* fluorescence and pigments content were found. Major genetic fidelity was assessed by flow cytometry to measure DNA content and DNA-ploidy and no differences were found between the two groups of plants. These data support that the micropropagation protocol induced no major DNA content changes.

Keywords: genetic stability, micropropagation, plant performance, photosynthesis, slash pine

Introduction

Slash Pine (*Pinus elliottii* var. *elliottii*) has particular economical value to timber industry due to its wood quality, and to its fast growth rates compared with other conifers. Moreover, this species is also characterized by the high quality resin production (Burns et al. 1991; Newton et al. 1995; Jain & Gupta 2005). *P. elliottii* var. *elliottii* was already introduced in reforestation programs and industrial plantations of this species can now be found worldwide, particularly in North America, Brazil or Australia.

Any breeding program of a selected species must include cloning strategies (e.g. Pascoe 2002) of the elite genotypes. Vegetative propagation techniques have been on the basis of the establishment of clonal seed orchards (CSO). In the last decades *in vitro* plant propagation, namely micropropagation, has been successfully applied to forestry species for the mass propagation of important and economically valuable forest species (e.g. Pinto et al. 2002; Conde et al. 2008), including pine species (e.g. Tang et al. 2001; Pullman et al. 2003; Lelu-Walter et al. 2006; Tang et al. 2006; Marum et al. 2009; Klimaszewska et al. 2011). Micropropagation allows the conservation of forest species, as it made possible an acceleration of plant physiological rejuvenation acceleration, and a germplasm rescue, regardless of the availability of seed (Aguiar et al. 2011). This is particularly important for forest elite genotypes in species that have long life cycles, as is de case of most *Pinus* species. In particular for *Pinus*, acceleration of the growth rates and biomass production may be a crucial economical gain.

The successful application of micropropagation techniques on a commercial scale requires that this technique provide genetically stable plantlets (e.g. Conde et al. 2004; Lopes et al. 2006; Fernandes et al. 2008) with good physiological performance (e.g. Santos et al. 2003) obtained with low cost and high survival rates. Also major constraints may arise during acclimatization (often associated with, e.g. root dysfunctions, stomatal and gas exhange disorders), which may impact carbon metabolism and ultimately plant growth. In particular, typical *in vitro* propagation conditions such as high humidity, low illumination and CO_2 levels, high carbon source levels and the presence of growth regulators, provide minimal stress and optimum conditions for shoot/plant multiplication, but may result in the development of morphologically, anatomically and physiologically abnormal plantlets

(Osório et al. 2012; Dias et al. 2013). The heterotrophism promoted during *in vitro* conditions and the poorly developed mechanisms to control water loss (e.g. poor stomatal control, cuticular abnormalities) render micropropagated plants vulnerable to the transplantation shocks when directly placed in a greenhouse or field. During acclimatization these abnormalities are corrected and plants adapt to the new growth conditions (Hazarika 2006; Dias et al. 2014). The ability to modify the phenotype and its underlying metabolism in response to environmental changes is known as phenotypic plasticity (Osório et al. 2013). So, to validate a micropropagation protocol there is a need to compare micropropagated and seed-derived plants in the same ambient conditions to evaluate the phenotypic plasticity of micropropagated plants and ensure that they can adapt to survive in their natural environment.

Physiological performance of micropropagated plants has been followed in several species, such as *Tuberaria major* (Osório et al. 2013), *Olea maderensis* (Santos et al. 2003), *Ceratonia siliqua* (Osório et al. 2012). In a few cases, differences in the performance of micropropagated plants were identified, but after a period of adaptation to the natural environment performances were mostly identical to those from plants originating from seed. For *Pinus taeda* no differences in the physiological or morphological performance between somatic seedlings and zygotic seeds were found (Becwar & Pullman 1995). However, when plants were grown in drought-prone sites, significant differences were found between the performances of seedlings versus micropropagated plantlets, but their magnitude was small and decreased in time (sixth and seventh growing seasons) (Rahman et al. 2003). These authors supported that plantations on regions prone to drought periods need to use more developed/elder loblolly pine plants when these have an *in vitro* origin (Rahman et al. 2003).

It has been described that the stability of plant genomes can be affected by the *in vitro* conditions to which the plants are subjected during the propagation process. Due to the possible occurrence of somaclonal variation the analysis of the ploidy stability of micropropagated plants is of particular importance. FCM is one of the most reliable techniques to estimate the DNA ploidy level and nuclear DNA content in plants (e.g. Leal et al. 2006; Loureiro et al. 2007a; Brito et al. 2008; Marum et al. 2009). In comparison with other methods, as Feulgen microdensitometry and chromosome counting, FCM provides unsurpassed ease, speed and accuracy (Doležel & Bartoš 2005). In recent years,

this technique has been successfully applied in the analysis of somaclonal variation in a vast number of woody species (Endemann et al. 2001; Pinto et al. 2004; Fernandes et al. 2008), including conifers (Libiakova et al. 1995; O'Brien et al. 1996; Aderkas et al. 2003; Loureiro et al. 2007a; Marum et al. 2009).

We have recently developed a protocol for micropropagation of *P. elliottii* (described in chapter II.1)(Figure 2) suitable for mass propagation of elite genotypes. Despite protocols have been developed for other conifers and *Pinus* in particular (e.g. Tereso et al. 2006; Kalia et al. 2007; Oliveira et al. 2012), no published work is available on the functional performance of micropropagated plantlets under *ex vitro* conditions in comparision with plants derived from seedlings. The aim of this work was to determine that he micropropagation protocol developed for slash pine did not compromise the performance of the micropropagated plants, so not jeopardising biomass production. For that, leaf gas exchange, chlorophyll fluorescence characteristics, relative water content and pigments and carbohydrates quantification in micropropagated and seed source plants were analyzed. The ploidy stability of plantlets and seedlings was assessed using FCM.

Materials and Methods

Plant Material and experimental conditions

Seeds obtained from open pollinated trees, of *Pinus elliotti* var *eliiotti* (PE), were provided by the company Resisul Fortaleza, Ltda.. Seeds were collected in São Paulo region, Brazil at 2009 and conserved at 4ºC until utilization. These seeds were used to the control (seedlings germinated *ex vitro* on the greenhouse, Figure 1A) and as explants for the production of micropropagated plantlets (Figure 1B). Plantlets used in this study were obtained from micropropagated *in vitro* culture collection of PE previously established from shoot apices of seedlings as described in chapter II.1 and summarized in Figure 2.

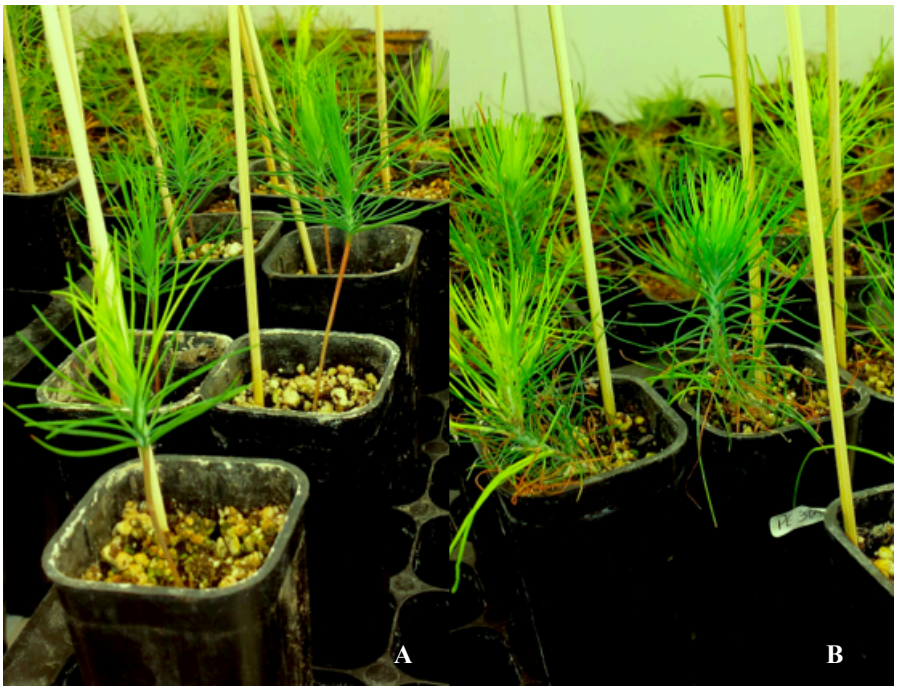

Figure 1 – *P. elliottii* var. *elliottii* plants used on this study: A - seedlings germinated *ex vitro*; B - micropropagated plantlets.

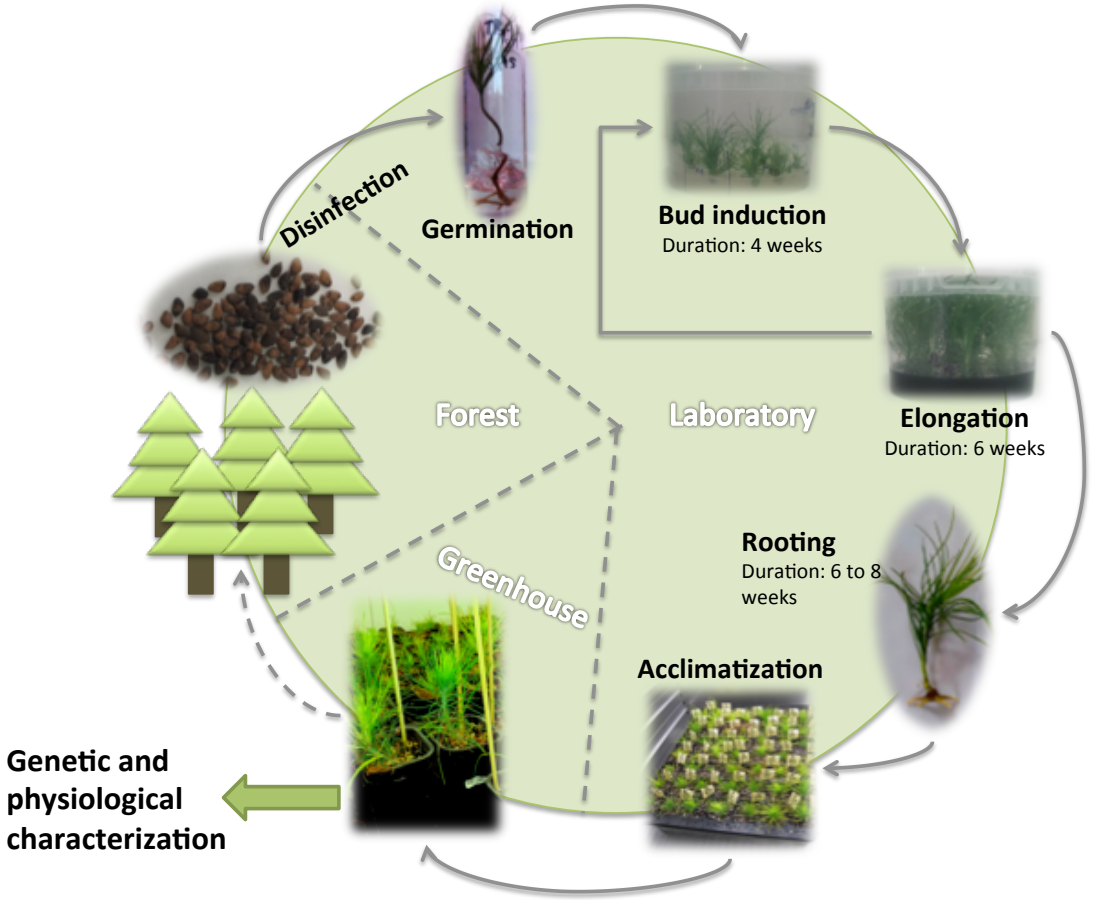

Figure 2 - Summary of micropropagation protocol developed for *Pinus elliottii* var. *elliotti* and described on chapter II.1: Briefly, seeds were germinated and after four weeks shoot apices of seedlings were inoculated in WV5 medium (Coke 1996) enriched with 10 µM BAP. After, 4 weeks shoots were transfered to an

elongation medium (WV5+0,2% activated charcoal (AC)). Rooting was achieved after 6-8 weeks in WV5 medium + 10 μM IBA. Plants were acclimatized then with success.

Groups of plantlets and seedlings (each group with seven individuals) were used in this study. Both seedlings and micropropagated plantlets had approximately 8 cm of shoot height and average age around 6±1 months. Plantlets from micropropagation were placed, one month prior to the start of the assay, in a growth chamber with a day/night temperature of 27/22ºC, a 16-h photoperiod at a light intensity of approximately 300 μmol $m^{-2}s^{-1}$ and relative humidity (%RH) of 50%.

Plant water status

Plant water status was assessed by assessing the relative water content (RWC) of needle segments. RWC was calculated as 100*(FW-DW)/(TW-DW), where FW is the fresh weight of the needles, TW is their turgid weight (determined after floating for 180 min on distilled water at 5 °C in dark) and DW is the dry weight (determined after drying the needle segments at 80 °C for 1 week).

Quantification of pigments and carbohydrates

Chlorophyll (Chl) and carotenoids were extracted with a acetone/Tris (50 mM pH 7.8) buffer (80:20, v:v) and measured by spectrophotometric absorption at 470, 537, 647 and 663 nm. Pigment contents were calculated as described by Sims and Gamon (2002).

Soluble sugars and starch were extracted from leaf samples and quantified using the anthrone method described by Irigoyen et al. (1992) and Osaki et al. (1991), respectively.

Photosynthesis measurements

Chlorophyll *a* fluorescence features were measured *in situ* with a pulse-amplitude-modulated fluorimeter (FMS 2, Hansatech Instruments, Norfolk, England). Maximum quantum efficiency of photosystem II (PSII) was calculated as $F_v/F_m = (F_m - F_0)/F_m$ by measuring the fluorescence signal from a group of dark-adapted needles when all reaction centers are open using a low intensity pulsed measuring light source (F_0) and during a

pulse saturating light (0.7 s pulse of 5,000 lmol photons m^{-2} s^{-1} of white light) when all reactions centers are closed (F_m). Following F_v/F_m estimation, after a 20s exposure to actinic light (500 μmol m^{-2} s^{-1}), light-adapted steady-state fluorescence yield (F_s) was averaged over 2.5 s, followed by exposure to saturating light (5,000 μmol m^{-2} s^{-1}) for 0.7 s to establish F_m'. The sample was then shaded for 5 s with a far-red light source to determine F_0' which was used for determination of photochemical quenching, $q_P = (F'_m - F_s)/(F'_m - F'_0)$, non-photochemical quenching NPQ = $(F_m-F'_m)/F'_m$) and the effective photochemical efficiency of PSII, $\Phi_{PSII} = \Delta F/F'_m = (F'_m - F_s)/F'_m$ (Dias et al. 2013).

In situ CO_2 assimilation rate (*A*) and transpiration rate (*E*) were determined in intact needles with a portable infrared gas analyzer (LC_{pro+}, ADC, Hoddesdon, UK), operating in open mode under growth chamber conditions, according to von Caemmerer and Farquhar (1981) equations. Measurements were always performed in the youngest fully developed needles in the growth conditions: daily photoperiod at growth temperature (26°C) and atmospheric CO_2 concentration.

Flow cytometric analyzes

PE needles were used as source material for analyzing putative changes in DNA (DNA content and ploidy stability) and in cell cycle dynamics. Nuclear suspensions were obtained from approximately 50 mg of plant material according to the protocol previously described by Loureiro et al. (Loureiro et al. 2007a,b). In brief, nuclei were released from cells by chopping with a razor blade in 1 mL of Woody Plant Buffer (WPB) (0.2 M Tris–HCl, 4 mM $MgCl_2$., 2 mM EDTA Na_2, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7.5). For ploidy analysis PE needles were chopped together with *Vicia faba* cv. Inovec (2C = 26.90 pg DNA) leaves, used as internal reference standard. To minimize release of cytosolic compounds, chopping was quick (less than 30 s) and not very intense. Nuclear suspension was filtered with a 50 μm nylon mesh. After that, nuclei were stained with 50 μg/mL propidium iodide (*Pi*, Fluka) and to avoid *Pi* staining of RNA, 50 μg/mL of RNAse (Sigma, St. Louis, USA) was also added to nuclei suspension. Nuclei were analyzed in an Attune® Acoustic Focusing Cytometer (Life Technologies Applied Biosystems, Vic, Australia) where data was acquired using the

Attune® Software (version 1.2.5, Life Technologies Applied Biosystems). In each replicate, at least 5,000 nuclei were analyzed (7 plants per condition were analyzed).

The ploidy levels (and putative occurrence of aneuploidy or polyploidy) were determined by analyzing the G_0/ G_1 peaks position and/or appearance of new G_0/G_1 peaks. For each sample, the DNA content was calculated as the ratio between mean fluorescence of the G_0/G_1 peak of sample and internal standard. The nuclear DNA content of PE was further estimated by multiplying the DNA index by the known genome size of the internal standard, i.e., 2C=26.90pg. This mass value was converted into total number of base pairs using the formula 1pg = 978Mbp (Doležel et al. 2003). The CV value of G_0/G_1 peaks, as a measure of fluorescence dispersion was also recorded. For cell cycle analysis, the proportion of cells in each cell cycle phases (G_0/G_1, S and G_2) was determined.

Raw data were exported as Static Dimension data files LMD files (Listmode File Format) and analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA) and when necessary data were transformed to achieve normality and equality of variance. When these criteria were not satisfied even with transformed data, the non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks was performed. The post hoc analysis was evaluated by Holm-Sidak method. The significance level was 0.05. All statistical analysis was performed using SigmaPlot for Windows, version 11.0.

Results

After one month on the same conditions, PE micropropagated plantlets and seedlings with approximately the same age and similar morphology were evaluated for plant water status by the measurement of the RWC. No significant differences were found in the RWC between the two groups of plants (Figure 3A).

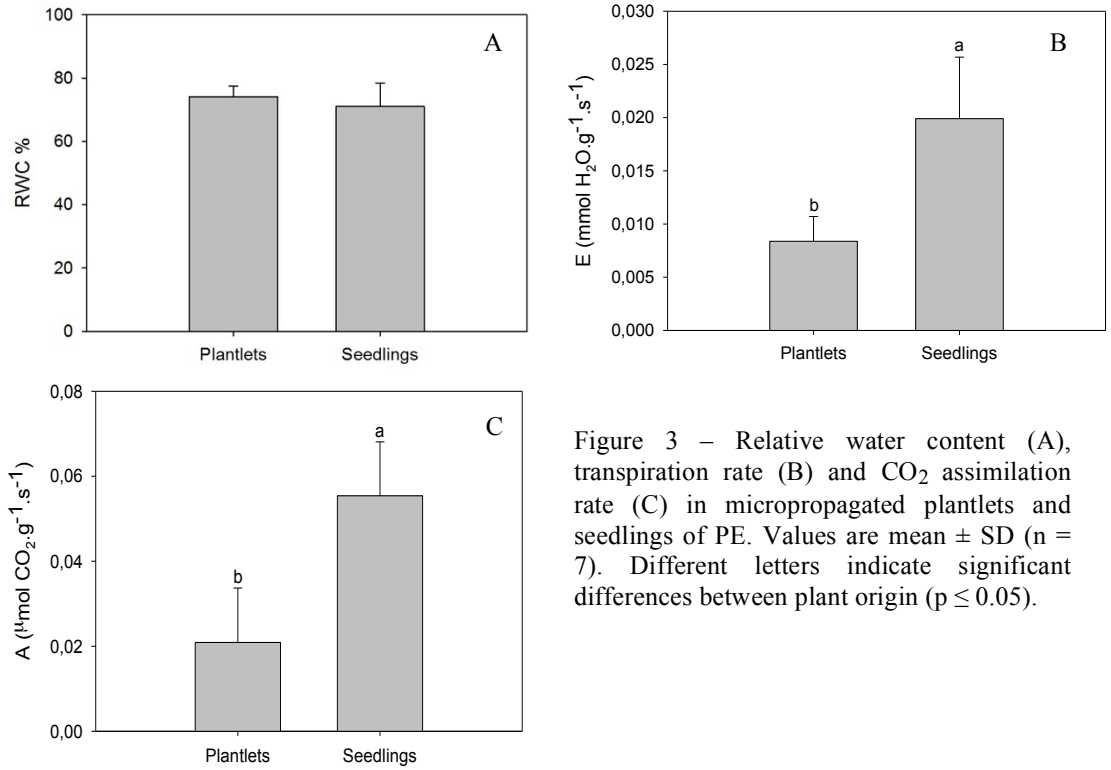

Figure 3 – Relative water content (A), transpiration rate (B) and CO_2 assimilation rate (C) in micropropagated plantlets and seedlings of PE. Values are mean ± SD (n = 7). Different letters indicate significant differences between plant origin ($p \leq 0.05$).

However, for the gas exchange parameters, seedlings showed significantly higher ($p \leq 0.05$) transpiration rate (*E*) and CO_2 assimilation rate (*A*) than plantlets (Figure 3B and C). Seedling values for both parameters reached ~2 and ~3 times the values of plantlets, respectively for *E* and *A*.

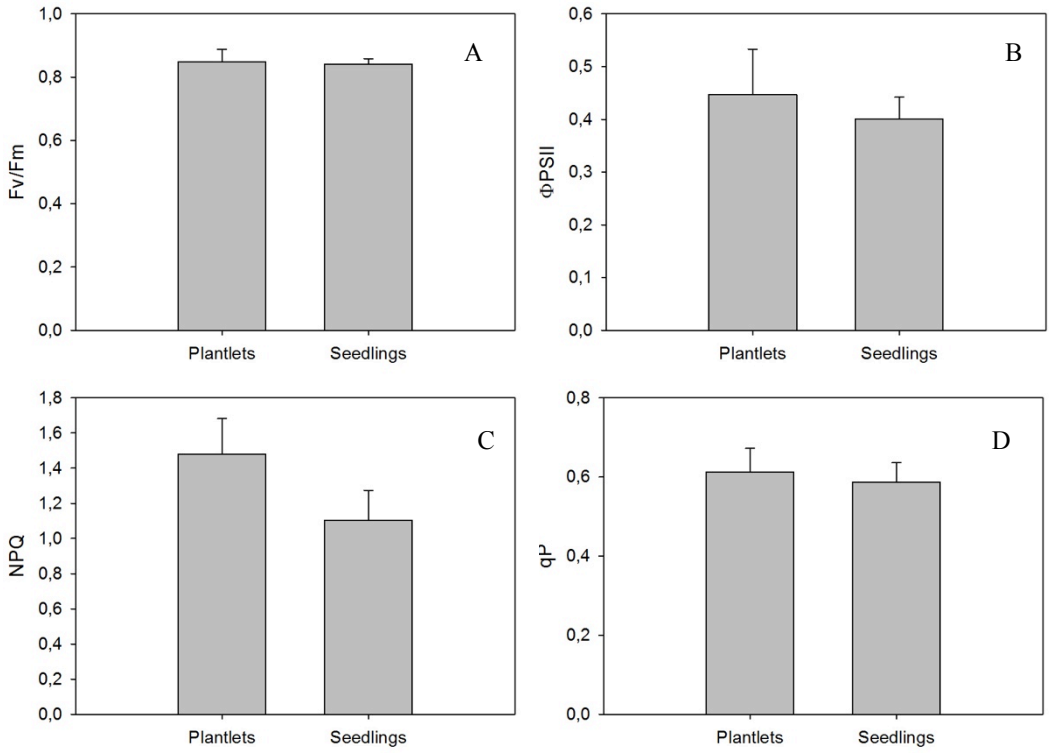

Figure 4 - Maximum quantum yield of photosystem II (F_v/F_m) (A), quantum yield of PSII (FPSII) (B), photochemical quenching (qP) (C) and non-photochemical quenching (NPQ) (D) in micropropagated plantlets and seedlings of PE. Values are mean ± SD (n = 7). For these data no significant differences were found ($p > 0.05$).

Concerning the chlorophyll *a* fluorescence parameters, similar values ($p \leq 0.05$) of F_v/F_m, Φ_{PSII}, qP and NPQ were obtained in micropropagated plantlets and seedlings of PE (Figure 4).

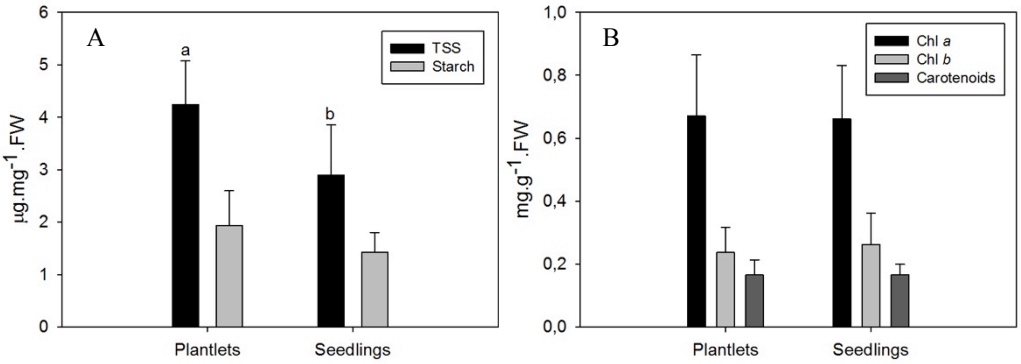

Figure 5 – Carbohydrates content (A) and pigments content (B) in micropropagated plantlets and seedlings of PE. Values are mean ± SD (n = 7). Different letters indicate significant differences between plant origin ($p \leq 0.05$).

The concentration of TSS was significantly higher in micropropagated plantles than in seedlings, but no significant differences were observed in the starch content (Figure 5A). Also, the concentrations of the photosynthetic pigments were simliar in PE plantlets and seedlings (Figure 5B).

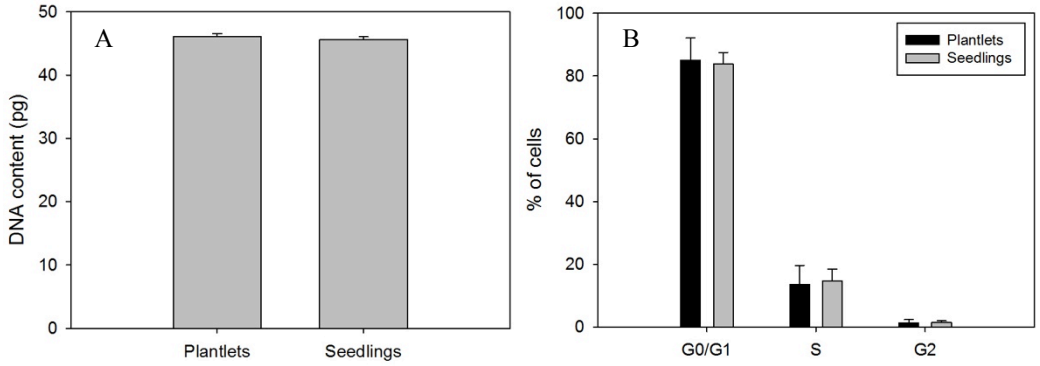

Figure 6 – Nuclear DNA content (A) and cell cycle dinamics (B) for micropropagated plantlets and seedlings of PE. Values are mean ± SD (n = 7). For these data no significant differences were found ($p > 0.05$).

Considering the DNA content, the mean nuclear DNA content ($2C_{PE}/2C_{Vicia\ faba}$ x *Vicia faba* DNA content) obtained for the micropropagated plantlets (46.05 pg) was similar ($p > 0.05$) to the one obtained for the seedlings (45.63 pg) (Figure 6A). The cell cycle analysis showed also, no significant differences in the cell cycle phases between the two groups of plants (Figure 6B).

Discussion

Commercial use of micropropagation is still limited in many species due to the high mortality rates that occur during acclimatization (e.g. Hazarika 2006; Pospíšilová et al. 2009), mostly due to anatomic-functional abnormalities developed during *in vitro* growth (Hazarika 2006). From the comparative studies between *Pinus elliotii* plants with similar ages and growth conditions, but with different origins (micropropagated vs seedling), we demonstrate in this study that micropropagated plants accomplishe satisfactorily our acclimatization protocol and achieve photosynthetic performances similar to seedlings. Our data also suggest that after six months of acclimatization the stomatal system of PE plantlets is already functional, helping to regulate the *E*. This contributes to the healthy plant water status of PE.

Concerning photosynthesis, PE seedling plants showed a higher CO_2 assimilation rate than plantlets. During acclimatization PE plants minimized the *E* to avoid dehydration, but the reduction of the stomatal aperture can limit CO_2 exchange. So, a putative lower internal CO_2 availability in the intercellular spaces of the mesophyll cells can be one of the causes of the lower *A* in PE plantlets. Also RuBisCO activity has been pointed as one of the major constraints in *in vitro* culture plants during the acclimatization process. A low amount and activity of RuBisCO has been reported as one of the main causes of reduced photosynthetic rates in *in vitro* plants (Dias et al. 2013; Dias et al. 2014). Lower levels of *A* were also found in micropropagated trees of *Ulmus glabra*, but the authors argued that this fact does not necessarily mean slower height or stem diameter growth rates (Ďurkovič et al. 2010).

Chlorophyll *a* fluorescence has become one of the most powerful techniques to assess photochemical efficiency and photoinhibition and it has been widely used (Bolhar-Nordenkampf et al. 1989) to monitor plant performance during the acclimatization (Dias et al. 2011; Dias et al. 2013; Osório et al. 2013). Both PE plantlets and seedlings have a similar photosynthetic efficiency. Moreover, the F_V/F_m ratio in plantlets and seedlings are in the range of 0.75–0.85 meaning that plants are healthy and unstressed. Similarly, no differences in the ΦPSII and qP was found for both groups. All these data support that the lower *A* of PE plantlets are not related to photochemical limitation (e.g. ATP and NADPH availability).

Relatively to non-photochemical processes, the parameter NPQ is an indicator of thermal dissipation in PSII antennae and in a typical plant its value should range between 0.5 and 3.5 at saturating light intensities (Maxwell & Johnson 2000). PE plantlets and seedlings present values inside this range. However, PE plantlets showed a tendency to higher NPQ (despite not significant) indicating that some excess energy is being dissipated protecting plantlets photosynthetic photoaparatus from photoinhibition. Consequently, less light energy is being used for photosynthesis and lower *A* can be found in PE plantlets.

Chlorophyll and carotenoids act as light-harvesting pigments. Moreover, carotenoids can also play an important role in preventing oxidative damage. According to Amâncio et al. (1999), high sugar concentration in the *in vitro* culture medium may inhibit chlorophyll

synthesis. However, in our study, similar levels of pigment contents in PE plantlets and seedlings strongly support that PE plantlets soon reached autotrophy.

Concerning the carbohydrate levels, PE plantlets have more TSS sugars than their seedling counterparts, but both groups had similar levels of starch. The higher levels of TSS in PE plantlets can even be attributed to the *in vitro* growth conditions (culture medium with high levels of sucrose). Also, high levels of sugars are usually related to a sugar-mediated source-sink feedback inhibition of CO_2 assimilation or due to a reduction of sucrose hydrolysis due to changes in invertase activity (Podazza et al. 2006). A decrease in RuBisCO activity seems to be correlated with sugars accumulation in leaves. Roh and Choi (2004) demonstrated that in *in vitro* tobacco plants, the highest RuBisCO activity was achieved when grown at 4% sucrose, but for higher sucrose concentrations, RuBisCO activity was substantially reduced. Moreover, although *in vitro* plantlets may appear normal, they may fail to be actively photosynthesizing, due to the exogenous supply of sucrose, which makes unnecessary the normal development of photosynthetic apparatus (Hazarika 2006; Dias et al. 2013). Taking all these findings into consideration, the TSS accumulation in plantlets may induce a sugar-mediated feedback inhibition decreasing RuBisCO activity and concomitant reducing the *A*.

The nuclear DNA content of *Pinus elliottii* var. *elliottii* had been previously estimated by other authors (Table 1), and although it is assumed that the comparison of the data obtained in different laboratories may have some constraints due to the use of different reference standards (Doležel & Bartoš 2005; Loureiro et al. 2007a), the DNA content values obtained with the present work were very similar to those reported previously with FCM. Only the value published by Ohri and Khoshoo (1986) is significantly lower but was determined by Feulgen microdensitometry. These results strongly indicate that the micropropagation protocol used to establish PE apparently does not induce major DNA-content changes and that the plantlets obtained through this protocol were geneticaly uniform. Also the cell cycle analysis showed that the micropropagation protocol did not affect cell cycle dynamics of PE plant leaves. These findings confirm that plants regenerated from well-developed meristematic tissues have reduced tendency for genetic variation (Kanchanapoom et al. 2012).

Other authors have used flow cytometry to validate micropropagation protocols. In case of *Jatropha curcas* and *Anubias barteri* each source of plant material tested gave the same ploidy level and showed no variation when compared to the mother plants (Kaewpoo & Te-Chato 2010; Kanchanapoom et al. 2012). On the other hand, Franco et al. (2014) found a variation in ploidy level in one of the *Jatropha curcas* genotypes propagated by organogenesis. In fact, it is known that the clones derived from organogenesis from callus have a higher risk of mutation than the ones derived from axillary buds (Kalia et al. 2007). For *Pinus pinaster* it was reported that the mean DNA content obtained in the different somatic embryogenesis-derived tissues, using the somatic embryogenesis protocol described by Marum et al. (2009), was similar to the value obtained for zygotic embryos from the same species.

Table 1 - Estimations of nuclear DNA content for *P. elliottii* obtained in this work and by other authors.

Tissue	DNA content (pg/2C)	Technique[c]	Protocol[d]	Reference
Needles of micropropagated plantlets	46.05	*Pi*:FCM	*Vicia faba* (2C = 26.90 pg); WPB buffer	Present work
Needles of seedlings	45.63	*Pi*:FCM	*Vicia faba* (2C = 26.90 pg); WPB buffer	Present work
Root tips	35.27	Fe	*Allium cepa* (2C = 33.55 pg)	(Ohri & Khoshoo 1986)
Megagametophyte[a]	44.72	*Pi*:FCM	*P. eldarica* (2C = 47.30 pg) and *Hordeum vulgare* (2C = 11.12 pg); modified Galbraith and Michaelson buffer	(Wakamiya et al. 1993)
Megagametophyte[a]	45.22	*Pi*:FCM	*Pisum sativum* (2C = 8.22 pg); modified Galbraith and Michaelson buffer	(Williams et al. 2002)
Megagametophyte[b]	49.38	*Pi*:FCM	*Hordeum vulgare* (2C = 11.12 pg); modified Galbraith buffer	(Grotkopp et al. 2004)

[a] The nuclear DNA content was estimated for haploid tissues

[b] The nuclear DNA content reported by Grotkopp et al. (2004) was estimated considering a DNA content ratio between diploid and haploid tissues of 2.

[c] Fe—Feulgen microdensitometry; *Pi*:FCM—flow cytometry using propidium iodide.

[d] Protocol description includes reference standard (with genome size) and nuclear isolation buffer.

Conclusions

Due to its enormous industrial importance, breeding programs are being developed to both improve *P. elliottii* and clone elite genotypes. For that, micropropagation protocols are crucial, but require that regenerated plants undergo a correct acclimatazation stage, and have good functional performance. Our data indicate that both the micropropagation protocol previously developed by our group produced plantlets that during acclimation stage had most of the functional performances close to those of seed derived plants. Minor differences were found only regarding lower *A* values shown by plantlets, which were probably due to the lower *E* (low CO_2 availability), deficient RuBisCO activity or due to a sugar-mediated feed-back inhibition. Moreover, the micropropagation methodology used did not induce major genetic changes in the micropropagated plantlets and the primary goal of "true-to-type" propagation was attained.

References

Aderkas, P. von, R. Pattanavibool, K. Hristoforoglu, and Y. Ma. 2003. "Embryogenesis and Genetic Stability in Long Term Megagametophyte-Derived Cultures of Larch." *Plant Cell, Tissue and Organ Culture* 75 (1). Kluwer Academic Publishers: 27–34. doi:10.1023/A:1024614209524.

Aguiar, Ananda Virgínia de, Valderês Aparecida de Sousa, Elenice Fritzsons, and José Elidney Pinto Junior. 2011. "Programa de melhoramento de pinus da Embrapa Florestas." Embrapa Florestas.

Amâncio, Sara, João Paulo Rebordão, and Maria Manuela Chaves. 1999. "Improvement of Acclimatization of Micropropagated Grapevine: Photosynthetic Competence and Carbon Allocation." *Plant Cell, Tissue and Organ Culture* 58 (1). Kluwer Academic Publishers: 31–37. doi:10.1023/A:1006323129593.

Becwar, Michael R, and Gerald S Pullman. 1995. "Somatic Embryogenesis in Loblolly Pine (*Pinus taeda* L.)." In *Somatic Embryogenesis in Woody Plants*, 287–301. Springer.

Bolhar-Nordenkampf, H R, S P Long, N R Baker, G Oquist, ULEG Schreiber, and E G Lechner. 1989. "Chlorophyll Fluorescence as a Probe of the Photosynthetic Competence of Leaves in the Field: A Review of Current Instrumentation." *Functional Ecology*, 497–514.

Brito, Gina, João Loureiro, Tina Lopes, Eleazar Rodriguez, and Conceição Santos. 2008. "Genetic Characterisation of Olive Trees from Madeira Archipelago Using Flow Cytometry and Microsatellite Markers." *Genetic Resources and Crop Evolution* 55 (5): 657–64. doi:10.1007/s10722-007-9268-8.

Burns, J A, O J Schwarz, and S E Schlarbaum. 1991. "Multiple Shoot Production from Seedling Explants of Slash Pine (*Pinus elliottii*, Engelm.)." *Plant Cell Reports* 10 (9): 439–43. doi:10.1007/BF00233810.

Conde, P, J Loureiro, and C Santos. 2004. "Somatic Embryogenesis and Plant Regeneration from Leaves of *Ulmus minor* Mill." *Plant Cell Reports* 22 (9): 632–39. doi:10.1007/s00299-003-0735-1.

Conde, Paula, Alexandra Sousa, Armando Costa, and Conceição Santos. 2008. "A Protocol for *Ulmus minor* Mill. Micropropagation and Acclimatization." *Plant Cell, Tissue and Organ Culture* 92 (1): 113–19. doi:10.1007/s11240-007-9310-8.

Dias, M. C., C. Correia, J. Moutinho-Pereira, H. Oliveira, and C. Santos. 2014. "Study of the Effects of Foliar Application of ABA during Acclimatization." *Plant Cell, Tissue and Organ Culture (PCTOC)* 117 (2): 213–24. doi:10.1007/s11240-014-0434-3.

Dias, M. C., G. Pinto, C. M. Correia, J. Moutinho-Pereira, S. Silva, and C. Santos. 2013. "Photosynthetic Parameters of *Ulmus minor* Plantlets Affected by Irradiance during Acclimatization." *Biologia Plantarum* 57 (1): 33–40. doi:10.1007/s10535-012-0234-8.

Dias, M. C., G. Pinto, and C. Santos. 2011. "Acclimatization of Micropropagated Plantlets Induces an Antioxidative Burst: A Case Study with *Ulmus minor* Mill." *Photosynthetica* 49 (2): 259–66. doi:10.1007/s11099-011-0028-9.

Doležel, J, J Bartoš, H Voglmayr, and J Greilhuber. 2003. "Nuclear DNA Content and Genome Size of Trout and Human." *Cytometry Part A* 51: 127–28. doi:10.1002/cyto.a.10013.

Doležel, Jaroslav, and J A N Bartoš. 2005. "Plant DNA Flow Cytometry and Estimation of Nuclear Genome Size." *Annals of Botany* 95 (1): 99–110.

Ďurkovič, Jaroslav, Ingrid Čaňová, Tibor Priwitzer, Mária Biroščíková, Pavol Kapraľ, and Milan Saniga. 2010. "Field Assessment of Photosynthetic Characteristics in Micropropagated and Grafted Wych Elm (*Ulmus glabra* Huds.) Trees." *Plant Cell, Tissue and Organ Culture (PCTOC)* 101 (2): 221–28. doi:10.1007/s11240-010-9680-1.

Endemann, M., K. Hristoforoglu, T. Stauber, and E. Wilhelm. 2001. "No Title." *Biologia Plantarum* 44 (3). Kluwer Academic Publishers: 339–45. doi:10.1023/A:1012426306493.

Fernandes, P., E. Rodriguez, G. Pinto, I. Roldan-Ruiz, M. De Loose, and C. Santos. 2008. "Cryopreservation of *Quercus suber* Somatic Embryos by Encapsulation-Dehydration and Evaluation of Genetic Stability." *Tree Physiology* 28 (12): 1841–50. doi:10.1093/treephys/28.12.1841.

Franco, Mariana Crotti, Daniela de Argollo Marques, Walter José Siqueira, and Rodrigo rocha Latado. 2014. "Micropropagation of *Jatropha curcas* Superior Genotypes and Evaluation of Clonal Fidelity by Target Region Amplification Polymorphism (TRAP) Molecular Marker and Flow Cytometry." *AFRICAN JOURNAL OF BIOTECHNOLOGY* 13 (38): 3871–80. doi:10.5897/AJB14.13649.

Grotkopp, Eva, Marcel Rejmánek, Michael J. Sanderson, and Thomas L. Rost. 2004. "Evolution of Genome Size in Pines (*Pinus)* and Its Life-History Correlates: Supertree Analyzes." *Evolution* 58 (8). The Society for the Study of Evolution: 1705. doi:10.1554/03-545.

Hazarika, B.N. 2006. "Morpho-Physiological Disorders in in Vitro Culture of Plants." *Scientia Horticulturae* 108 (2): 105–20. doi:10.1016/j.scienta.2006.01.038.

Irigoyen, J. J., D. W. Einerich, and M. Sanchez-Diaz. 1992. "Water Stress Induced Changes in Concentrations of Proline and Total Soluble Sugars in Nodulated Alfalfa (Medicago Sativd) Plants." *Physiologia Plantarum* 84 (1): 55–60. doi:10.1111/j.1399-3054.1992.tb08764.x.

Jain, S. Mohan, and Pramod K. Gupta, eds. 2005. *Protocol for Somatic Embryogenesis in Woody Plants*. Dordrecht: Springer Netherlands. doi:10.1007/1-4020-2985-3.

Kaewpoo, M, and S Te-Chato. 2010. "Study on Ploidy Level of Micropropagated *Jatropha curcas* L. via Flow Cytometry." *Journal of Agricultural Technology* 6 (2): 391–400.

Kalia, R. K., S. Arya, S. Kalia, and I. D. Arya. 2007. "Plantlet Regeneration from Fascicular Buds of Seedling Shoot Apices of *Pinus roxburghii* Sarg." *Biologia Plantarum* 51 (4): 653–59. doi:10.1007/s10535-007-0138-1.

Kanchanapoom, Kantamaht, Panyaros Chunui, and Kamnoon Kanchanapoom. 2012. "Micropropagation of *Anubias barteri* var. *nana* from Shoot Tip Culture and the Analysis of Ploidy Stability." *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*. doi:10.15835/nbha4027520.

Klimaszewska, Krystyna, Catherine Overton, Don Stewart, and Robert G Rutledge. 2011. "Initiation of Somatic Embryos and Regeneration of Plants from Primordial Shoots of 10-Year-Old Somatic White Spruce and Expression Profiles of 11 Genes Followed during the Tissue Culture Process." *Planta* 233 (3): 635–47. doi:10.1007/s00425-010-1325-4.

Leal, F, J Loureiro, E Rodriguez, M S Pais, C Santos, and O Pinto-Carnide. 2006. "Nuclear DNA Content of *Vitis vinifera* Cultivars and Ploidy Level Analyzes of Somatic Embryo-Derived Plants Obtained from Anther Culture." *Plant Cell Reports* 25 (9): 978–85. doi:10.1007/s00299-006-0162-1.

Lelu-Walter, MA, M Bernier-Cardou, and K Klimaszewska. 2006. "Simplified and Improved Somatic Embryogenesis for Clonal Propagation of *Pinus pinaster* (Ait.)." *Plant Cell Reports* 25 (8): 767–76. doi:10.1007/s00299-006-0115-8.

Libiakova, G., A. Gajdosova, B. Vookova, M. Kubincova, and J. Dolezel. 1995. "Karyological Study of *Abies concolor* X *Abies grandis* Calli and Shoots Regenerated in Vitro." *Biologia (Slovakia)*.

Lopes, T, G Pinto, J Loureiro, A Costa, and C Santos. 2006. "Determination of Genetic Stability in Long-Term Somatic Embryogenic Cultures and Derived Plantlets of Cork Oak Using Microsatellite Markers." *Tree Physiology* 26: 1145–52.

Loureiro, J., A. Capelo, G. Brito, E. Rodriguez, S. Silva, G. Pinto, and C. Santos. 2007a. "Micropropagation of *Juniperus phoenicea* from Adult Plant Explants and Analysis of Ploidy Stability Using Flow Cytometry." *Biologia Plantarum* 51 (1): 7–14. doi:10.1007/s10535-007-0003-2.

Loureiro, João, Eleazar Rodriguez, Jaroslav Dolizel, and Conceição Santos. 2007b. "Two New Nuclear Isolation Buffers for Plant DNA Flow Cytometry: A Test with 37 Species." *Annals of Botany* 100: 875–888. doi:10.1093/annbot/mcm152.

Marum, Liliana, João Loureiro, Eleazar Rodriguez, Conceição Santos, M. Margarida Oliveira, and Célia Miguel. 2009. "Flow Cytometric and Morphological Analyzes of *Pinus pinaster* Somatic Embryogenesis." *Journal of Biotechnology* 143 (4): 288–95. doi:10.1016/j.jbiotec.2009.08.001.

Maxwell, K., and G Johnson. 2000. "Chlorophyll Fluorescence-a Practical Guide." *Journal of Experimental Botany* 51 (345): 659–68. doi:10.1093/jexbot/51.345.659.

Newton, RJ, KA Marek-Swize, ME Magallanes-Cedeno, N Dong, S Sen, and SM Jain. 1995. "Somatic Embryogenesis in Slash Pine (*Pinus elliottii* Engelm.)." In *Somatic Embryogenesis in Woody Plants, Volume 3 - Gymnosperms*, edited by SM Jain, PK Gupta, and RJ Newton, 183–95. doi:10.1007/978-94-011-0960-4_11.

O'Brien, IEW, DR Smith, RC Gardner, and BG Murray. 1996. "Flow Cytometric Determination of Genome Size in *Pinus*." *Plant Science* 115 (1): 91–99. doi:10.1016/0168-9452(96)04356-7.

Ohri, D., and T. N. Khoshoo. 1986. "Genome Size in Gymnosperms." *Plant Systematics and Evolution* 153 (1-2): 119–32. doi:10.1007/BF00989421.

Oliveira, LF, LLF Ribas, M Quoirin, HS Koehler, E Amano, and AR Higa. 2012. "Micropropagation of *Pinus taeda* L. from Juvenile Material." *Tree and Forestry Science and Biotechnology* 6 (1): 96–101.

Osaki, Mitsuru, Takuro Shinano, and Toshiaki Tadano. 1991. "Redistribution of Carbon and Nitrogen Compounds from the Shoot to the Harvesting Organs during Maturation in Field Crops." *Soil Science and Plant Nutrition* 37 (1). Taylor & Francis Group: 117–28. doi:10.1080/00380768.1991.10415017.

Osório, M. L., S. Gonçalves, N. Coelho, J. Osório, and A. Romano. 2013. "Morphological, Physiological and Oxidative Stress Markers during Acclimatization and Field Transfer of Micropropagated *Tuberaria major* Plants." *Plant Cell, Tissue and Organ Culture (PCTOC)* 115 (1): 85–97. doi:10.1007/s11240-013-0343-x.

Osório, M. L., J. Osório, S. Gonçalves, M. M. David, M. J. Correia, and A. Romano. 2012. "Carob Trees (*Ceratonia siliqua* L.) Regenerated in Vitro Can Acclimatize Successfully to Match the Field Performance of Seed-Derived Plants." *Trees* 26 (6): 1837–46. doi:10.1007/s00468-012-0753-0.

Pascoe, S. 2002. "Somatic Embryogenesis In *Pinus radiata*." Faculty of Engineering and Science, Victoria University.

Pinto, G, J Loureiro, T Lopes, and C Santos. 2004. "Analysis of the Genetic Stability of *Eucalyptus*

globulus Labill. Somatic Embryos by Flow Cytometry." *Theoretical and Applied Genetics* 109 (3): 580–87. doi:10.1007/s00122-004-1655-3.

Pinto, G, H Valentim, A Costa, S Castro, and C Santos. 2002. "Somatic Embryogenesis in Leaf Callus from a Mature *Quercus suber* L. Tree." *In Vitro Cellular & Developmental Biology - Plant* 38 (6): 569–72. doi:10.1079/IVP2002352.

Podazza, G, M Rosa, J A González, M Hilal, and F E Prado. 2006. "Cadmium Induces Changes in Sucrose Partitioning, Invertase Activities, and Membrane Functionality in Roots of Rangpur Lime (*Citrus limonia* L. Osbeck)." *Plant Biology (Stuttgart, Germany)* 8 (5): 706–14. doi:10.1055/s-2006-924171.

Pospíšilová, J., H. Synková, D. Haisel, and P. Baťková. 2009. "Effect of Abscisic Acid on Photosynthetic Parameters during Ex Vitro Transfer of Micropropagated Tobacco Plantlets." *Biologia Plantarum* 53 (1): 11–20. doi:10.1007/s10535-009-0003-5.

Pullman, G S, S Johnson, G Peter, J Cairney, and N Xu. 2003. "Improving Loblolly Pine Somatic Embryo Maturation: Comparison of Somatic and Zygotic Embryo Morphology, Germination, and Gene Expression." *Plant Cell Reports* 21 (8): 747–58. doi:10.1007/s00299-003-0586-9.

Rahman, Mohd S., Michael G. Messina, and Ronald J. Newton. 2003. "Performance of Loblolly Pine (*Pinus taeda* L.) Seedlings and Micropropagated Plantlets on an East Texas Site." *Forest Ecology and Management* 178 (3): 257–70. doi:10.1016/S0378-1127(02)00483-8.

Roh, Kwang Soo, and Bok Youl Choi. 2004. "Sucrose Regulates Growth and Activation of Rubisco in Tobacco Leaves in Vitro." *Biotechnology and Bioprocess Engineering* 9 (3): 229–35. doi:10.1007/BF02942298.

Santos, Conceição V., Gina Brito, Gloria Pinto, and Henrique M.A.C. Fonseca. 2003. "In Vitro Plantlet Regeneration of *Olea europaea* Ssp. Maderensis." *Scientia Horticulturae* 97 (1): 83–87. doi:10.1016/S0304-4238(02)00148-6.

Sims, Daniel A, and John A Gamon. 2002. "Relationships between Leaf Pigment Content and Spectral Reflectance across a Wide Range of Species, Leaf Structures and Developmental Stages." *Remote Sensing of Environment* 81 (2-3): 337–54. doi:10.1016/S0034-4257(02)00010-X.

Tang, W, Z Guo, and F Ouyang. 2001. "Plant Regeneration from Embryogenic Cultures Initiated from Mature Loblolly Pine Zygotic Embryos." *In Vitro Cellular & Developmental Biology-Plant* 37 (5): 558–63. doi:10.1007/s11627-001-0097-8.

Tang, Wei, Ronald J Newton, and Thomas M Charles. 2006. "Plant Regeneration through Multiple Adventitious Shoot Differentiation from Callus Cultures of Slash Pine (*Pinus elliottii*)." *Journal of Plant Physiology* 163 (1): 98–101. doi:10.1016/j.jplph.2005.04.030.

Tereso, S, S Gonçalves, L Marum, M Oliveira, J Maroco, and C Miguel. 2006. "Improved Axillary and Adventitious Bud Regeneration from Portuguese Genotypes of *Pinus pinaster* Ait." *Propagation of Ornamental Plants* 6 (1): 24–33.

von Caemmerer, S, and G D Farquhar. 1981. "Some Relationships between the Biochemistry of Photosynthesis and the Gas Exchange of Leaves." *Planta* 153 (4): 376–87. doi:10.1007/BF00384257.

Wakamiya, I, RJ Newton, JS Johnston, and HJ Price. 1993. "Genome Size and Environmental Factors in the Genus *Pinus*." *American Journal of Botany* 80 (11): 1235–41.

Williams, Claire G., Karen L. Joyner, Lisa D. Auckland, Spencer Johnston, and H. James Price. 2002. "Genomic Consequences of Interspecific *Pinus* Spp. Hybridization." *Biological Journal of the Linnean Society* 75 (4): 503–8. doi:10.1046/j.1095-8312.2002.00038.x.

CHAPTER III

SOMATIC EMBRYOGENESIS IN HYBRID *PINUS ELLIOTTII* VAR. *ELLIOTTII X PINUS CARIBAEA* VAR. *HONDURENSIS* AND ASSESSEMENT TO TRUE-TO-TYPENESS

Chapter prepared to be submitted as an original paper to a SCI journal:

Nunes S, Marum L, Pereira VT, Almeida T, Farinha N, Sousa D, Mano N, Figueiredo J, Dias MC, Santos C. 2016. Somatic embryogenesis in hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis* and assessement to true-to-typeness.

Abstract

A somatic embryogenesis protocol that showed high reproducibility for different OP families of *P. elliottii x P. caribaea* (an interspecific *Pinus* hybrid with major economic value) is described. Different conditions were tested from the initiation to plant regeneration. Immature megagametophytes of five open pollinated (OP) mother trees were used for initiation during two consecutive years (2013 and 2014). Initiation rates were of 21.44 (in 2013) and 11.35% (in 2014), when explants were inoculated on the basal medium mLV supplemented with BAP (1 $mg.L^{-1}$) and 2,4-D (2 $mg.L^{-1}$). The mother tree genotype conditioned percentages of initiation, but the designed culture conditions allowed the establishment of embryogenic cell lines (ECLs) from all OP families on both years. Somatic embryos evolved to cotyledonary ones on maturation medium (basal mLVmedium supplemented with 40 μM ABA and 6% w/v sucrose), with a success rate of 52.4%. Plantlets' conversion was achieved with a success of 60 to 86%. Four months after acclimatization, plants survival rates ranged between 78 and 85%. True-to-typeness along the different phases of the SE process was demonstrated by flow cytometry analysis of the DNA-ploidy of the embryonic masses and emblings.

Keywords: Somatic embryogenesis, *Pinus elliottii x Pinus caribaea*, conifers, Multi-varietal Forestry, clonal propagation

Introduction

The interspecific hybrid between *Pinus elliottii* var. *elliottii* and *Pinus caribaea* var. *hondurensis* was first performed in 1955 in Australia. This hybrid combines the parents' favourable characteristics, providing superior growth and form, and is primarily used for structural timbers, veneer and plywood products (Nikles 2000; Shepherd et al. 2002). Due to its improved characteristics, the industrial demand for the *P. elliottii x P. caribaea* hybrid is increasing on sites traditionally planted with *P. elliottii* (e.g. South and Central America and South Africa).

Like its parents, this interspecific hybrid has been involved in breeding programs that aim at achieving commercial success in forestry by developing high-value clonal varieties. The application of biotechnology, such as micropropagation techniques, to forest tree breeding programs offers a great potential to hasten the pace of tree improvement for desirable end uses (Pinto et al. 2002; Campbell et al. 2003). Despite the potential of this tropical hybrid for commercial plantation forestry, little attention has been paid to establish biotechnological tools for *in vitro* culture and large-scale clonal propagation.

Among *in vitro* techniques, somatic embryogenesis (SE) offers unique advantages in forest breeding programs. This technology enables the implementation of multi-varietal forestry (MVF), which is defined as the use of tested tree varieties in a forest plantation. MVF offers several advantages when compared with conventional tree breeding programs like the possibility of capture greater genetic gain than is possible by seed orchard breeding, flexibility to rapidly deploy suitable varieties with changing breeding goals and ability to design genetic gain and diversity (Park et al. 2006; Park & Bonga 2011). Besides SE contribution for clonal selection and commercial production, this technique also allows the development of transgenics and the cryopreservation of embryogenic tissues to halt the physiological aging that affects the other clonal material (Cyr & Klimaszewska 2002; Marum et al. 2004; Fernandes et al. 2008). The SE in association with the cryopreservation of the embryogenic tissues is a key technology for implementing MVF, since it gives the opportunity to propagate the same genotypes consistently over time (Park & Bonga 2011). Cryopreservation has allowed long-term field testing of the clones produced and the selection of superior embryogenic cell lines (ECLs) prior to mass production (Bonga 2015).

SE is defined as a multi-step regeneration process starting with the initiation of embryogenic masses, followed by embryo maturation and plant regeneration (Conde et al. 2004; Carneros et al. 2009). SE initiation is influenced by various factors, like the plant genotype, the development stage of the initial explant, the type of explant itself and the induction media (Pinto, Silva, et al. 2008; Montalbán et al. 2011). Somatic embryogenesis techniques that have been established for pine species usually explants derived from juvenile material, like immature megagametophytes containing zygotic embryos. In fact these explants have been described as the most responsive explants for initiation of embryogenic masses in this genus (e.g. Klimaszewska et al. 2001; Pullman & Johnson 2002; Miguel et al. 2004; Stojičić et al. 2015; Humánez et al. 2012; Alvarez et al. 2013).

Several studies focused on improving initiation, proliferation and maturation stage through the study of many culture basal media nutrient formulations and plant growth regulators, trace elements and their combinations (Liao & Amerson 1995; Klimaszewska et al. 2001; Miguel et al. 2004; Park et al. 2006; Carneros et al. 2009; Humánez et al. 2012). Different species responded differently to the optimized media, enhancing the importance of the species/genotype specificity. A complete procedure for propagation by SE has been achieved in several pine species such as *P. radiata* (Montalbán et al. 2010), *P. taeda* (Tanget al. 2001), *P. pinaster* (Humánez et al. 2012; Alvarez et al. 2013), *P. sylvestris* (Park et al. 2006), *P. nigra* (Salajova & Salaj 2005), *P. banksiana* (Park et al. 2006), *P. strobus* (Klimaszewska et al. 2001; Park et al. 2006), *P. patula* (Ford et al. 2005), *P. pinea* (Carneros et al. 2009), and *P. halepensis* (Montalbán et al. 2013). Initiation of *P. elliottii* SE was first reported by Newton et al. (1995) who reported SE phases till germination, but with low rates of initiation and germination. Later, Pullman et al. (2005) published refinements of the SE initiation technology in *P. elliottii*. SE in *P. caribaea* was reported by David et al. (1995), although with low initiation rates. More recently, some SE protocols were published for interspecific conifer hybrids like the hybrids larches (Lelu-Walter & Pâques 2009) and *P. rigida x P. taeda* (Kim & Moon 2007).

Although SE offers multiple advantages, as stated above, the commercial application of this technology still presents a number of difficulties in the *Pinus* genus, such as a small window for embryogenic cell lines initiation (Miguel et al. 2004; Humánez et al. 2012), low initiation rates (Park et al. 2006; Carneros et al. 2009) and inhibition of the embryo development prior cotyledon emergence leading to low rates of complete mature somatic

embryos (Tang et al. 2001; Montalbán et al. 2010; Lara-Chavez et al. 2011). Also, difficulties during the differentiation of germinated somatic embryos (Newton et al. 1995) was reported. These constraints, together with the fact that SE protocols only have succeeded in a limited number of genotypes, imply that a reduced number of genotypes are putative candidates for clonal tests, which represents a serious limitation to the successful commercial application of this technology (Miguel et al. 2004; Montalbán et al. 2013).

To our knowledge, this is the first report on a robust SE of the *P. elliottii x P. caribaea* hybrid that was efficient for different elite families. Five open-pollinated (OP) families were examined for their capacity to undergo SE propagation. To achieve the described SE protocol, the influence of the genotype and culture media formulations on the initiation rates and maturation capacity of the ECLs was evaluated. Furthermore, a true-to-type propagation system using the optimized SE protocol was evaluated.

Materials and Methods

Plant Material

Immature cones from five open pollinated (OP) plus mother trees (families) of *Pinus elliotti* var. *elliotti x Pinus caribaea* var. *hondurensis* were collected in São Paulo region (Brazil) in the summer of 2013 and 2014. At least tree cones were collected from each mother tree. Cones were stored in plastic bags at 4°C for a maximum of 20 days, until immature seeds were dissected. Whole cones were brushed in 10% (v/v) commercial bleach with detergent (<5% active chloride), readily washed under running tap water and then disinfected for 15 min by immersion in a bleach solution with the same concentration, and rinsed three times in sterile water in the laminar flow bench. Afterwards, immature seeds were removed from the disinfected cones.

Initiation of somatic embryogenesis

For the initiation of embryogenic cultures of 2013 and 2014, megagametophytes with the enclosed immature zygotic embryos were aseptically removed from the seeds and used as explants.

In 2013 experiment, six different culture media were tested. Two basal medium formulations were selected from the literature on SE of *Pinus*, modified DCR medium (mDCR) based on DCR macro and microelements (Gupta & Durzan 1985) and MS vitamins (Murashige & Skoog 1962) with 0.1 $mg.L^{-1}$ tiamine and 100 $mg.L^{-1}$ myo-inosytol, and modified Litvay's (Litvay et al.1985) medium (mLV) with half-strength macroelements and full-strength microelements, vitamins and Fe-EDTA, as described by Klimaszewska et al. (2001). mDCR medium was supplemented with 250 $mg.L^{-1}$ L-glutamine (Duchefa Biochemie, DB) and 500 $mg.L^{-1}$ casein hydrolysate (DB), while mLV medium was supplemented with 500 $mg.L^{-1}$ L-glutamine and 1000 $mg.L^{-1}$ casein hydrolysate. All media contained 2% sucrose and 0.4% gellan gum (Gelrite, DB). Different plant growth regulators (PGR) combinations were tested with 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) or N-(2-Chloro-4-pyridyl)-N'-phenylurea (CPPU) as described on Table 1. The pH was adjusted to 5.8 before autoclaving. L-glutamine was filter-sterilized and added to the medium after autoclaving. In the 2013 experiment, six explants were incubated per petri dish (90x16mm) with at least 6 replicates per condition, while in the second year (2014), the megagametophytes were inoculated only in two culture media ES2 and ES5 (Table 1), with eight explants per petri dish, with 10 replicates per condition. The plates were incubated in the darkness conditions, at 23 ± 2 °C.

Table 1 - Composition of the culture media used for initiation of embryogenic cell lines.

Designation	Basal media		BAP	2,4-D	CPPU
	Macro/Micro	Vitamins	($mg.L^{-1}$)	($mg.L^{-1}$)	($mg.L^{-1}$)
ES1	DCR	vitMS	4	8	---
ES2	DCR	vitMS	1	2	---
ES3	DCR	vitMS	0.5	1	---
ES4	DCR	vitMS	---	---	1
ES5	mLV	vit.LV	1	2	---
ES6	mLV	vit.LV	---	---	1

Two months after inoculation, an extrusion embryogenic mass was observed from the megagametophytes. This embryogenic mass (EM) was analyzed by morphological and cytological observations. Extruded EM types were classified according to Liao and Amerson (1995), from Type 1 to Type 3 (see results). The number of initiated lines was recorded after four to eight weeks of culture under the conditions described below, for each OP tree family and medium tested.

Proliferation and maintenance of embryogenic masses

After four to eight weeks on initiation media, proliferating EM were separated from the megagametophyte, and subcultured bi-weekly on the same fresh medium (ES2 to ES6). The callus formed and initiated on ES1 was transferred to ES3 after 9 weeks. All the subcultures were maintained in dark, at 23 ± 1°C. An embryogenic cell line (ECL) was considered to be established when, after four months in proliferation medium, reached at least 1 g fresh weight.

Microscopic evaluations

The presence of embryogenic structures were confirmed and monitored in the different ECLs. The tissue samples were gently squashed in 2% acetocarmine and cytological observations were performed under light microscopy (Olympus BX53, Japan).

Maturation of somatic embryos

More than 50 ECLs induced in 2013 on the different media were screened for their capacity to produce mature somatic embryos. Two sets of experiments were carried out to assess the influence of genotype and abscisic acid (ABA) concentration in the maturation of somatic embryos (*Se*). For both maturation experiments the same procedure was used: 200 mg of embryogenic tissue, 7 days after subculture, was re-suspended in 3 ml of liquid proliferation medium, without PGR. Then 1 mL of cell suspension was spread on a filter paper (Whatman nº2, 55mm). Lastly the filter papers were placed on the surface of the maturation medium (MAT1 to MAT6). The ECLs that proliferated on DCR basal medium were transfered to the MAT1 to MAT3 media, while the ECLs proliferated in mLV basal medium were evaluated in the MAT4 to MAT6 media (Table 2). For each maturation condition at least 3 independent replicates were incubated for 10 weeks in a growth chamber, in the dark, at 22 ± 2 °C.

Table 2 - Composition of the culture media used for the maturation of somatic embryos.

Designation	Basal formulation	Vitamins	ABA (μM)
MAT1	DCR*	vitMS	40
MAT2	DCR*	vitMS	80
MAT3	DCR*	vitMS	120
MAT4	mLV**	vit.LV	40
MAT5	mLV**	vit.LV	80
MAT6	mLV**	vit.LV	120

Note: * DCR medium supplemented with 100 $mg.L^{-1}$ myo-inositol; 250 $mg.L^{-1}$ L-glutamine and 500 $mg.L^{-1}$ casein hydrolysate. ** mLV medium supplemented with 500 $mg.L^{-1}$ L-glutamine and 1000 $mg.L^{-1}$ casein hydrolysate. All media contain 6% sucrose and 0.9% gellan gum (Gelrite- DB).

Effect of ABA concentration

The ABA concentration was tested in 21 ECLs from the five families (A, B, C, D and E) used in the initiation experiments. Respecting the basal medium formulation each ECL was tested for its ability to produce mature somatic embryos on three different sequential ABA concentrations (40, 80 and 120 μM).

Effect of ECL genotype

The maturation performance was analyzed in 36 different ECLs, in order to study the effects of ECL genotype in the maturation capacity. The embryogenic tissue was inoculated on the maturation media MAT2 or MAT5 (Table 2), taking into account the two basal medium, mDCR or mLV from the proliferation stage.

Effect of proliferation culture medium

To access to the effect of initiation/proliferation medium on the maturation performance, 22 ECLs from different proliferation media (ES1 to ES6 – Table 1) were evaluated in the maturation media MAT2 or MAT5 (Table 2).

For the three experiments described above the somatic embryos differentiation and the following classification were performed according the stages defined by von Arnorld and Hakman (1988). The number of complete mature somatic embryos from stage III was quantified per fresh gram of embryogenic mass.

Plant conversion.

Isolated mature *Se* from two selected ECLs from the OP family B were transferred to half-strength basal medium (mDCR or mLV), without PGR, with 0.25% activated charcoal, 2% sucrose and solidified with 0.6% Gellan gum (Gelrite-DB). The *Se* were maintained one week in the dark, followed by one week in dim light and finally 3 weeks in normal light, in a growth chamber at 23 ± 2 °C, for a 16/8-h (day/night) photoperiod, under a photosynthetic photon flux density (PPFD) of approx. 50 ± 10 μmol m^{-2} s^{-1}. Afterwards, the germination rate was evaluated. The germinated embryos were then transferred to flasks with a culture medium equal to the germination medium, devoid of activated carbon, being maintained in the same conditions of temperature, light and photoperiod. About four weeks later, emblings with a well-developing epicotyle and hypocotyl (at least 3 cm long) were transferred to peat:perlite:vermiculite (8:1:1) and the conversion to plantlets rate was evaluated. At this point the root and shoot lengths and the presence of secondary roots were recorded. Emblings were then acclimatized in a growth chamber by gradually decreasing the relative humidity from 100 to 40% over a 10-day period (Conde et al. 2008). At the end of the acclimatization phase, emblings were transferred to the growth chamber and the survival rate was evaluated after 4 months.

True-to-typeness assessment by flow cytometry

Samples from EM under proliferation, emblings and mother trees from the OP families A, B and C were used as source material for analyzing putative changes in DNA (DNA content and ploidy stability). Nuclear suspensions were obtained from approximately 50 mg of plant material according to the protocol previously described by Loureiro et al. (2007 a,b). In brief, nuclei were released from cells by chopping with a razor blade in 1 mL of Woody Plant Buffer (WPB) (0.2 M Tris–HCl, 4 mM $MgCl_2$, 2 mM EDTA Na_2, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7.5). For ploidy analysis, EM or needles were chopped together with *Vicia faba* cv. Inovec leaves (2C = 26.90 pg DNA), used as internal reference standard. To minimize release of cytosolic compounds, chopping was quick (less than 30 s) and not very intense. Nuclear suspension was filtered with a 50 μm nylon mesh. After that, nuclei were stained with 50 μg.mL^{-1} propidium iodide (*Pi*, Fluka) and to avoid *Pi* staining of RNA, 50 μg.mL^{-1} of

RNAse (Sigma, St. Louis, USA) was also added to nuclei suspension (Brito et al. 2008). Nuclei were analyzed in a Attune® Acoustic Focusing Cytometer (Life Technologies Applied Biosystems, Vic, Australia) where data was acquired using the Attune® Software (version 1.2.5, Life Technologies Applied Biosystems). In each replicate, at least 5000 nuclei were analyzed.

The ploidy levels (and putative occurrence of aneuploidy or polyploidy) were determined by analyzing the G_0/ G_1 peaks position and/or appearance of new G_0/G_1 peaks. For each sample, the DNA content was calculated as the ratio between mean fluorescence of the G_0/G_1 peak of sample and internal standard. The nuclear DNA content of the samples was further estimated by multiplying the DNA index by the known genome size of the internal standard, i.e., 2C=26.90pg. This value was converted into total number of base pairs using the formula 1pg = 978Mbp (e.g. Loureiro et al. 2006). The CV value of G_0/G_1 peaks, as a measure of fluorescence dispersion was also recorded. Raw data were exported as Static Dimension data files LMD files (Listmode File Format) and analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA) and when necessary data were transformed to achieve normality and equality of variance. When these criteria were not satisfied even with transformed data, the non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks was performed. The post hoc analysis was evaluated by Holm-Sidak, Tukey or Student-Newman-Keuls methods. The significance level was 0.05. All statistical analyzes were performed using SigmaPlot for Windows, version 11.0.

Results

Initiation and establishment of ECLs

Initiation of embrogenic tissue was observed four to eight weeks after the megagametophytes inoculation (Figure 1A) on the initiation media tested in both 2013 and 2014 experiments (Table 3). The EM proliferation was initiated at the micropylar end of the megagametophyte (Figure 1B) showing a bright white or translucent mass with many

filaments. The three types of extruded EM described in Liao and Amerson criteria were observed: a type I EM (Figure 1B), consisting of a proliferative cell clump closely aggregated around the micropyle, a type II containing radially dispersed multiple small embryos typically not aggregated around the micropyle (data not shown) and a type III where the masses were produced away from the megagametophyte by a cord-like structure (data not shown). Acetocarmine staining of this white mass showed a typical embryogenic tissue, with aggregates of meristematic cells (small, round and densely cytoplasmic cells) together with long and highly vacuolated cells, forming a typical embryogenic tissue (Figure 1C). At the surface of the megagametophytes, a yellow compact tissue was also detected with a non-embryogenic *callus* aspect (data not shown). Only the EM proliferation was considered for the ECL initiation rate quantification.

In the experiment carried out in 2013, six different media with mDCR and mLV basal formulations and several PGR combinations with BAP, 2,4-D and CPPU were tested (Table 1). The average contamination rate was very low in this experiment (<1.3%).

The highest initiation rate mean was obtained in ES2 (20.13%) and ES5 (21.44%) media (Table 3), even though no significant differences ($p>0.05$) were detected between all media.

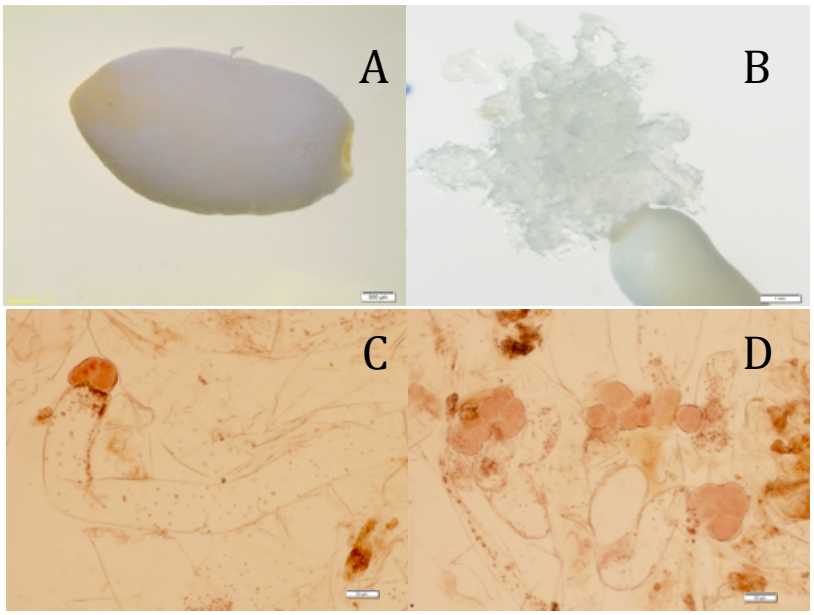

Figure 1 – Initiation of somatic embryogenesis in the hybrid *Pinus elliotti* var. *elliotti x Pinus caribaea* var. *hondurensis*. A – Isolated megagametophytes used as explant on the ECLs initiation (bar=500µm); B - Cell proliferation initiated at the micropylar end of the megagametophyte [Type I extrusion as described by Liao

and Amerson (1995)] (bar=1mm); C and D– Somatic pro-embryos with long suspensors observed in an established embryogenic line stained with acetocarmine (bar=50µm).

Table 3 - Mean of the initiation and establishment rates of ECLs in 2013 and 2014 years on each culture medium (ES1- ES6), from 5 OP families. Means ± SE (n=5). Data were analyzed by a one-way analysis of variance (ANOVA) and no significant differences were found.

Collection Year	Culture Medium	Number of inoculated explants	% Initiation ECL	% Establishment
2013	**ES1**	174	14.00 ± 6.43	3.00 ± 1.38
	ES2	178	20.13 ± 5.39	7.88 ± 4.52
	ES3	168	16.33 ± 3.21	6.33 ± 2.63
	ES4	180	2.78 ± 2.15	1.67 ± 1.67
	ES5	174	21.44 ± 5.55	10.22 ± 3.29
	ES6	174	5.56 ± 3.83	5.00 ± 3.77
2014	**ES2**	460	9.95 ± 4.65	6.10 ± 3.55
	ES5	414	11.35 ± 3.34	7.02 ± 2.14

The ECL establishment rates were determined 4 months after the induction of EM. The higher rate mean (10.2%) was also obtained in ES5 medium. Moreover, the ES5 was the only medium allowing the establishment of ECLs in all OP families (Figure 2). The use of higher concentrations of BAP and 2,4-D at the induction begining (four times higher in ES1 than in ES2) did not improve EM establishment. At the same time, the use of CPPU instead of BAP and 2,4-D (ES4 and ES6 media) was not benefitial for the majority of the OP families, since the ECL establishment was only possible in B and A/B families for ES4 and ES6, respectively (Table 3; Figure 2).

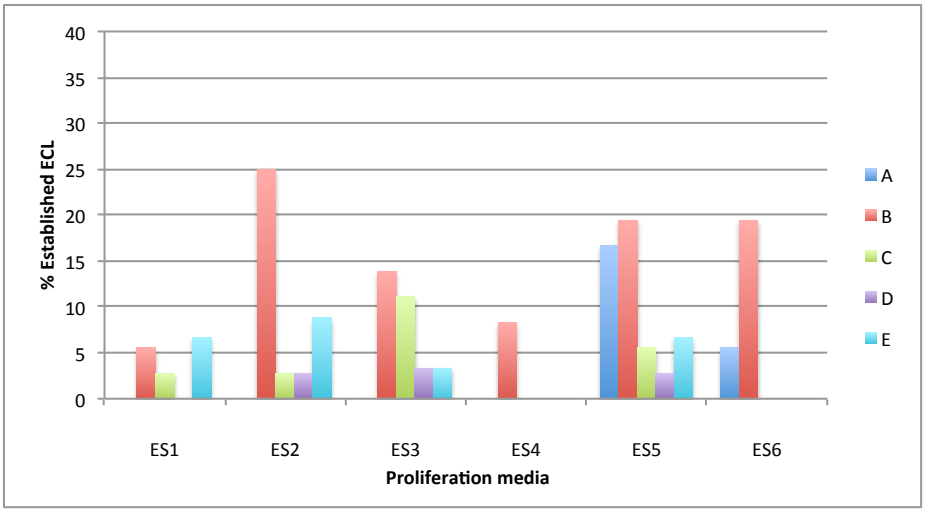

Figure 2 – ECL establishment rates on media ES1–ES6 obtained from the five OP mother trees (A – E), in 2013.

In the 2014 experiment, only the best initiation media (ES2 and ES5) obtained in 2013 were used in the five OP families. A decrease in initiation rate mean was observed compared to 2013 rates (Table 3). Taking into account, the different mother trees tested, the initiation rates in 2013 range from 11-33% in ES2 and 10-36% in ES5, while in 2014 the rates range from 0-28% in ES2 and 1.3-20% (Figure 3A).

In 2013 experiment, the families A and B had highest establisment rates, while in 2014, the family B presented a best performance, followed by A and E families with similar rates (Figure 3B).

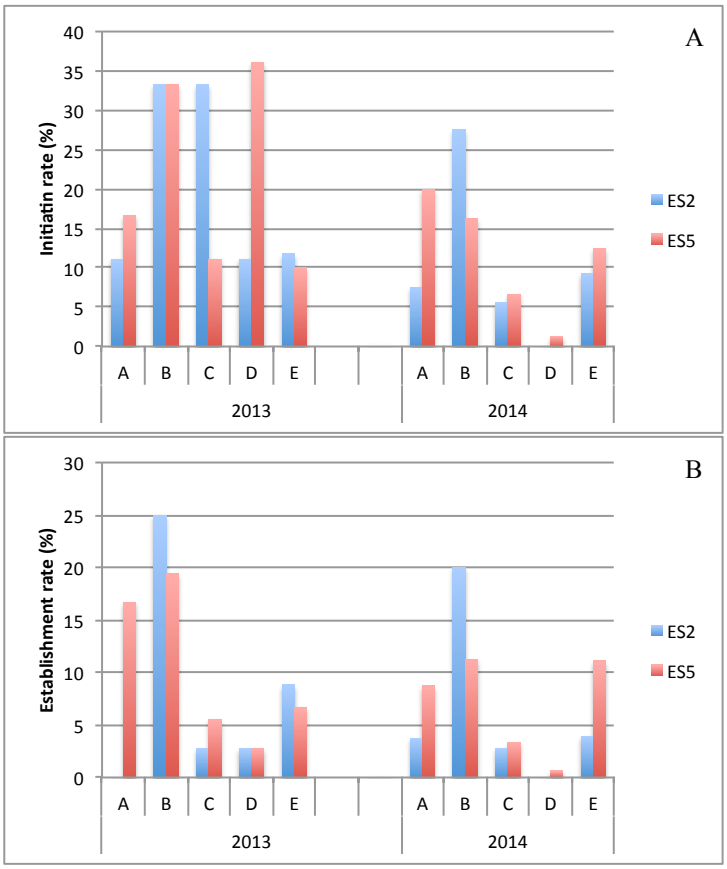

Figure 3 - Initiation (A) and establishment (B) ECLs rates on ES2 and ES5 media, in the five OP mother trees (A-E), from 2013 and 2014 experiments.

Maturation of somatic embryos

After 10 weeks of EM subculture, the pre-cotyledonary somatic embryos (stage II of Arnold and Hakman (1988) criteria) and the fully developed, cotyledonary *Se* (stage III) were differentiated from 50 different genotypes, isolated and quantified in each maturation medium analyzed (Figure 4).

To evaluate the influence of the ECL genotype on the *Se* ability to mature, 36 ELCs from the family B were selected and exposed to 80 μM of ABA. More than 47% of the ECLs tested produce mature *Se*, corresponding to the cotyledonary somatic embryos (stage III), after 10 weeks on maturation medium (Figure 4C). Pre-cotyledonary *Se* (stage II) were also observed on 39% of the ECLs tested (Figure 4B). The ECL genotype had a strong influence on the development of mature somatic embryos produced by gram of fresh weight of EM ($p \leq 0.05$) (Figure 5). The number of produced cotyledonary somatic embryos ranged from 5 to 915.4 mature *Se* / g FW, according to the ECLs tested.

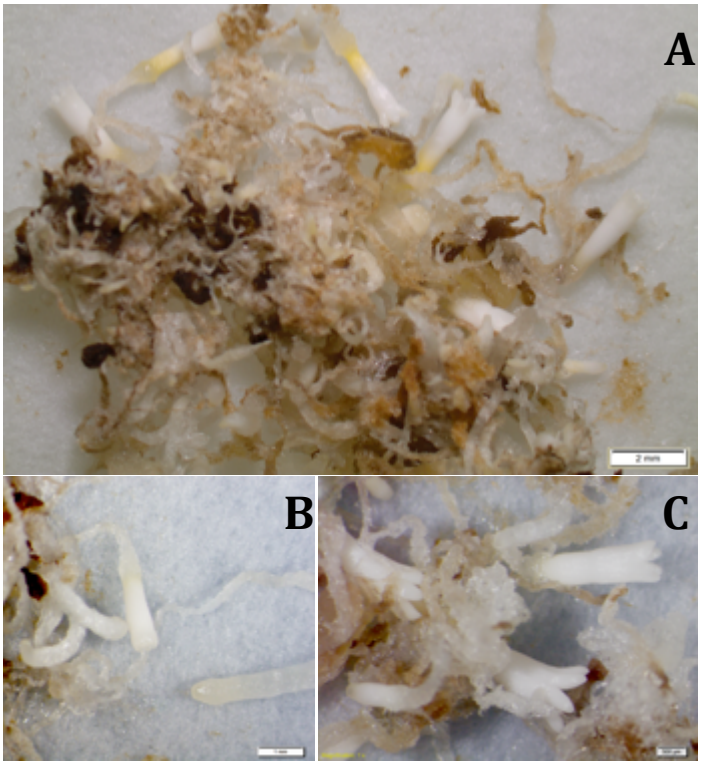

Figure 4 –Differentiation of somatic embryos during maturation of P. elliottii x P. caribaea. A – Se at different stages of development, after 10 weeks under maturation conditions (bar=2mm); B – Stage II somatic embryos (bar=1mm); C – Stage III somatic embryos (bar=500μm).

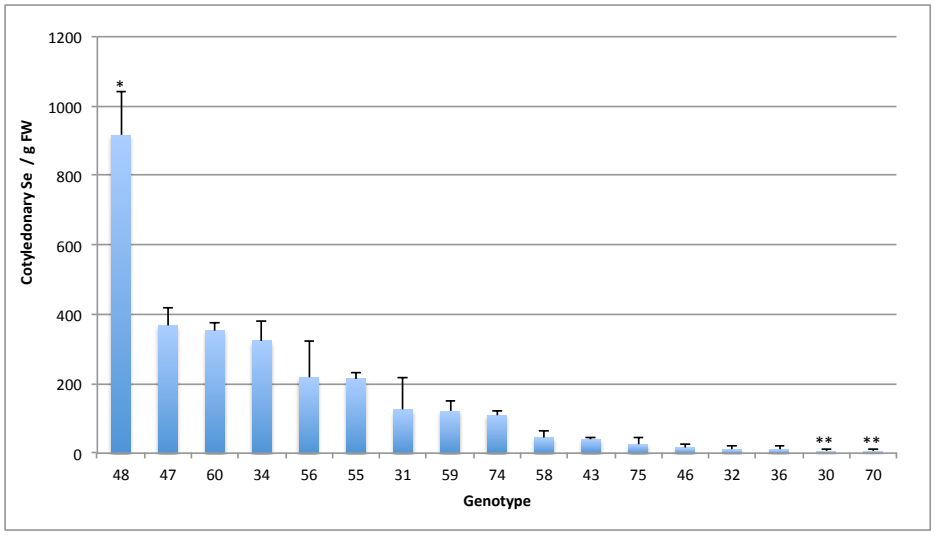

Figure 5 – Number of cotyledonary somatic embryos (Se) per gram of fresh weight of embryogenic tissue in the different ECLs established, from the mother tree B. Means ± SE ($n \geq 3$). ECLs marked with a * have significant differences, according to the Tukey's Test ($p \leq 0.05$).

The effect of ABA concentration on the maturation ability was evaluated in 21 selected ECLs, from the five OP plus families. The complete differentation of the cotyledonary embryos was quantified in the three ABA treatments (40, 80 and 120 μM) analyzed.

Cotyledonary *Se* (stage III) produced with 40, 80 and 120 μM ABA were obtained in 52.4%, 42.9% and 47.6% of the ECLs tested, respectively. The mean number of mature *Se* produced in families A, B, C and E is described in Figure 6. In OP A, B and C families (Figure 6-A, B and C) no differences were observed between the different treatments, on the number of mature *Se* produced. Nevertheless, there is a tendency to increase the number of mature *Se* produced with the decrease of ABA concentration in six ECLs (A.26; B.33; B.74; C.28; E.43; E.52). In OP E family (Figure 6D), one of the ECL (E.52) produced a significant higher number of mature *Se* on the ABA concentration of 40 μM (318.41 mature *Se* / g FW), than in 120 μM (34.83 mature *Se*/ g FW) ($p<0.05$). It is noteworthy that the line C.28 matured only in the culture medium with 40 μM ABA (Figure 6C).

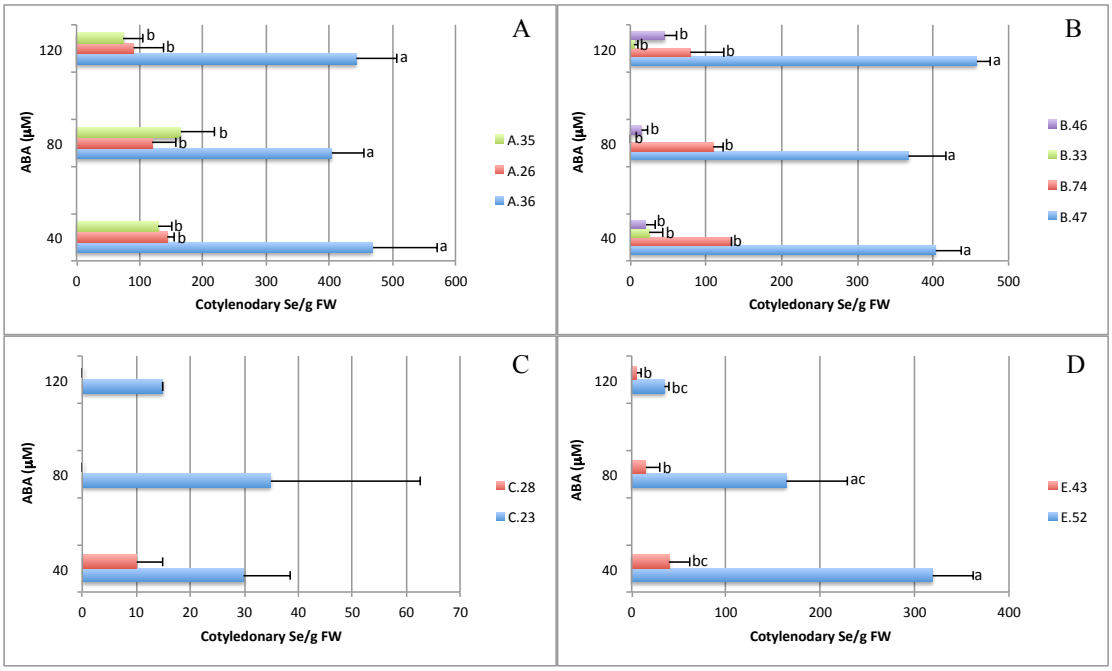

Figure 6 - Number of cotyledonary somatic embryos (*Se*) per gram of fresh weight of embryogenic tissue in function of ABA concentration. A – Family A; B – Family B; C – Family C; D – Family E. Means ± SE ($n \geq 3$). ECLs marked with different letters have significant differences, according to the Tukey's Test or Holm-Sidak Method ($p \leq 0.05$).

Taking into account the best ABA concentration obtained, 40 µM, the maturation rate and the number of mature *Se* per OP families (A-E) were analyzed. The higher maturation rate, 67%, was observed in family B. However, significant differences of the number *Se* /gFW were only obtained between family A and C (Table 4).

Table 4 – Maturation rate and number of cotyledonary somatic embryos (*Se*) per gram of fresh weight of EM obtained on the ABA concentration 40 µM per OP families. Means ± SE. Different letters have significant differences, according to the Dunn's Method ($p \leq 0.05$).

OP Family	Number of ECL	Maturation rate (%)	Number of cotyledonary *Se* / g FW
A	5	60.0 ± 24.5	148.3 ± 49.1 a
B	5	66.7 ± 18.3	116.4 ± 40.9 ab
C	5	33.3 ± 21.1	8.0 ± 3.5 b
E	4	41.7 ± 25.0	89.6 ± 41.4 ab

The influence of the proliferation medium on maturation ability was analyzed in 22 ECLs submitted to the 80 µM ABA maturation medium. The lines that were initiated and proliferated in the ES5 medium produced a significantly higher number of mature *Se* per gram of fresh weight than the ECLs proliferated in the ES1, ES2, e ES4 media ($p>0.05$)

(Table 5). Also, ECLs that proliferated on the basal medium mLV (ES5 and ES6) had a better maturation performance than those on mDCR.

Table 5 - Number of cotyledonary somatic embryos (*Se*) per gram of fresh weight of EM concerning the proliferation medium. Means ± SE ($n \geq 6$). Different letters have significant differences, according to the Holm-Sidak Method ($p \leq 0.05$).

Proliferation medium	**Number of cotyledonary *Se* / g FW**	
ES1	42.29 ± 25.31	b
ES2	94.53 ± 42.19	bc
ES4	9.95 ± 4.98	b
ES5	320.90 ± 54.95	a
ES6	156.72 ± 36.49	ac

Plant Conversion

To promote germination and conversion to plantlets, the cotyledonary somatic embryos were subcultured initially in darkness followed by dim light and finally at light conditions. The elongation of hypocotyls and radicule formation was observed after approximately 4-6 weeks (Figure 7B). In a first experiment performed with 40 mature *Se* per ECL, the germination rate was around 83% and 78% for B.48 and B.47 genotypes, respectively (Table 6). Thereafter, it was possible to observe the plantlet development with the epicotyl alongation and root formation of germinated somatic embryos, after 4-6 weeks (Figure 7C). Plantlet conversion rates recorded in the first experiment were around 50% (genotype B.48) and 22% (genotype B.47). A second experiment with a higher number of mature *Se* per ECL (N greater than 170) was performed and higher plantlet conversion rates were obtained with 86% and 60% for the genotypes B.48 and B.47, respectively. This improvement was due to the development and selection of germinated somatic embryos with a normal morphology, hypocotyl alongation and radicule formation, with more than 2 cm long (Table 6).

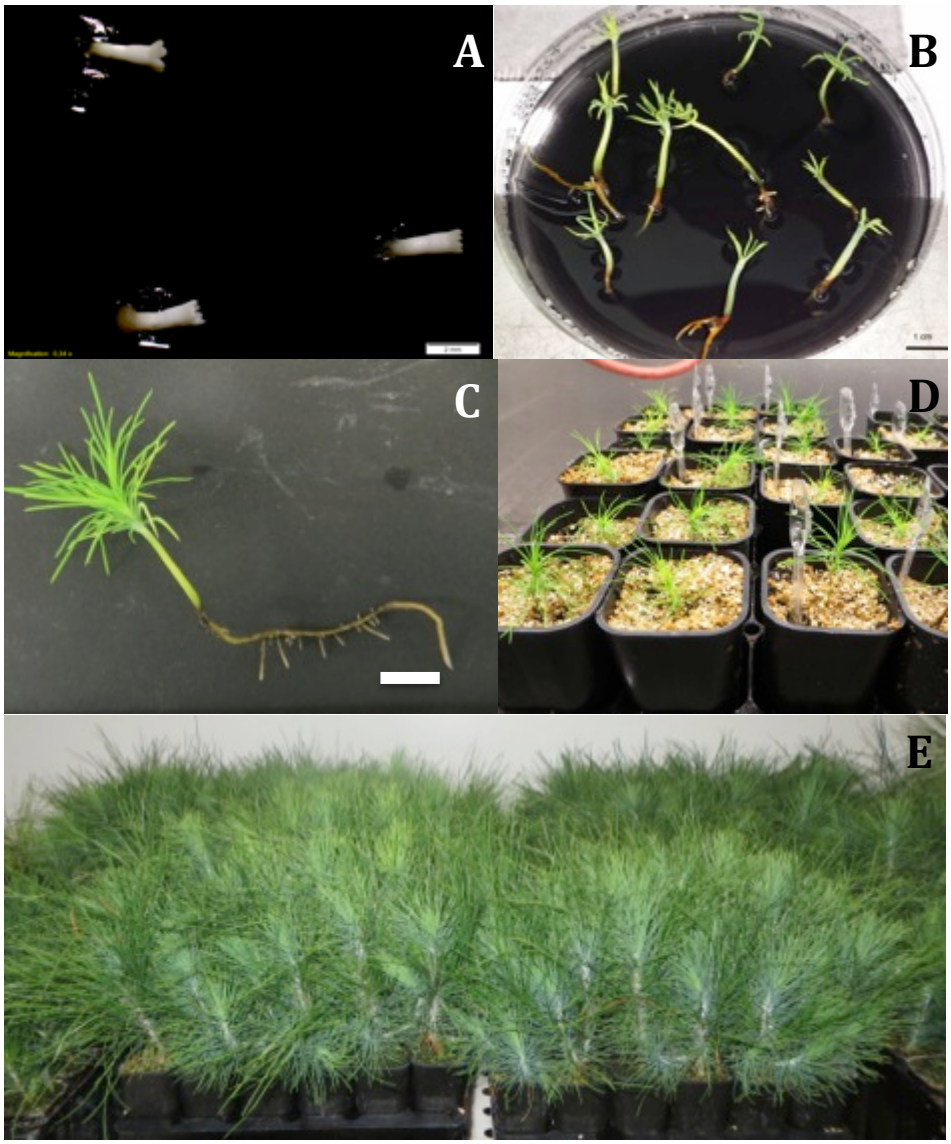

Figure 7 – Plantlet conversion and acclimatization of the Hybrid P. elliottii x P. caribaea. A – Cotyledonary *Se* at the beginning of the germination phase (bar=2mm); B – Somatic embryo conversion (bar=1cm); C – Emblings produced by SE 4-6 weeks after germination with the epicotyl elongated and formed roots (bar=1cm); D – Emblings on the acclimatization phase; E – Emblings after 5 months, in the greenhouse.

Table 6 – Germination and plantlets conversion rates obtained on the assays I and II, with cotyledonary Se produced by the two ECLs (B.47 and B.48).

Experiment	ECL	Number of cotyledonary *Se*	Germinated *Se* (%)	Plantlets conversion (%)
I	B.48	40	82.5	50.0
	B.47	40	77.5	22.5
II	B.48	178	88.2	86.0
	B.47	201	69.7	59.7

After 4 months in the greenhouse, the survival rates of the emblings were recorded (Table 7; Figure 7E). The higher survival rate achieved for the genotype B.48 (85%), could be

related to the better root development observed at the beginning of the acclimatization period. About 90% of the emblings from B.48 ECL had secondary roots, when compared to B.47 ECL.

Table 7 – Survival rate after 4 months in the greenhouse and data collected at the beginning of the acclimatization (root and aerial part lengths and rate of emblings with secondary roots) for the two ECLs analyzed. Means ± SE. n ≥ 9. Different letters have significant differences according to the Holm-Sidak Method ($p \leq 0.05$).

ECL	Survival * (%)	Root length (cm)	Shoot length (cm)	Secondary roots (%)
B.48	85	4.58 + 0.59 a	4.6 + 0.21 a	90.00
B.47	78	7.67 + 0.98 b	3.56 + 0.47 b	33.33

Genetic stability analysis

The ploidy level of OP mother trees A, B and C was assessed by flow cytometry (FCM), using leaves as nuclei source, and compared with 3 and 15 month-old EM from the same families. Histograms showed diploid profiles in all samples, similar to the profiles of mother plants (Figure 8A and B). Mother tree tissues were ranged between 1.7-1.74 pg/2C with minimal differences among genotypes. EM had a slight increase though not significant ($p>0.05$) that tended to increase with time (Table 8). With respect to emblings, with the exception of A.36 clones, DNA content values obtained for the emblings B.47 and B.48 are similar to those obtained for the mother trees ($p>0.05$) (Table 9). Once again no ploidy changes were observed in the emblings when compared to mother trees (Figure 8A and C).

Table 8 – Nuclear DNA content of the hybrid OP mother trees and embryogenic masses (EM) in proliferation for 3 and 15 months. Means ± SD. n ≥ 2. Data from the OP family B were analyzed by a one-way analysis of variance (ANOVA) and data from the OP families A and C were analyzed by a non-parametric Kruskal-Wallis ANOVA on Ranks, and no significant differences were found.

OP Family		DNA index	Nuclear DNA content (pg/2C)	CV (%)
A	Mother Tree	1.70 ± 0.01	45.60 ± 0.22	6.04
	EM 3 months	1.82 ± 0.01	48.97 ± 0.15	7.30
	EM 15 months	1.86 ± 0.04	50.11 ± 1.02	7.44
B	Mother Tree	1.74 ± 0.06	46.69 ± 1.71	6.64
	EM 3 months	1.80 ± 0.02	48.47 ± 0.51	6.21
	EM 15 months	1.78 ± 0.02	47.84 ± 0.45	7.64
C	Mother Tree	1.73 ± 0.00	46.46 ± 0.00	6.99
	EM 3 months	1.81 ± 0.02	48.56 ± 0.42	6.83
	EM 15 months	1.90 ± 0.01	51.12 ± 0.20	7.47

Table 9 - Nuclear DNA content of the hybrid OP mother trees and emblings from the OP families A and B. Means ± SD ($n \geq 2$). Different letters have significant differences, according to the Dunn's Method ($p \leq 0.05$).

	DNA index	Nuclear DNA content (pg/2C)	CV(%)
Mother tree A	1.70 ± 0.01 b	45.60 ± 0.22 b	6.04
Mother tree B	1.74 ± 0.06 b	46.69 ± 1.71 b	6.64
Emblings A.36	1.84 ± 0.05 a	49.36 ± 1.43 a	5.39
Emblings B.47	1.72 ± 0.01 b	46.26 ± 0.32 b	3.91
Emblings B.48	1.76 ± 0.03 b	47.27 ± 0.77 b	3.97

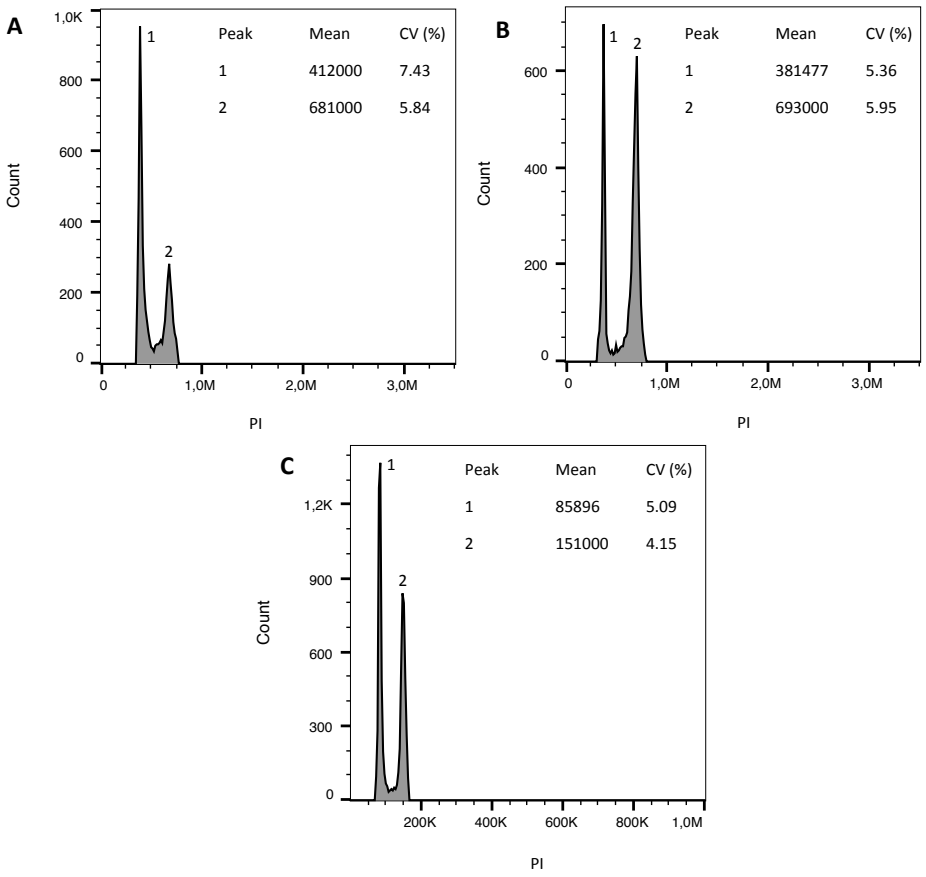

Figure 8 - Histograms of relative fluorescence intensity (PI) obtained for the hybrid *P. eliottii x P. caribaea*: A) from OP mother tree A; B) from 3 months old EM; C) from emblings A.36. Peak 1 – *Vicia faba*; Peak 2 – hybrid *P. eliottii x P. caribaea*. CV – coefficient of variation.

Discussion

In this study a successful protocol of SE was established from immature zygotic embryos of *P. elliotti* var. *elliotti* x *P. caribaea* var. *hondurensis*, with the set up of all the phases from initiation until acclimatization. To our knowledge, this is the first report in SE for this tropical hybrid. Some works of SE in *Pinus elliottii* (Liao & Amerson 1995; Newton et al.

1995) and *P. caribaea* (David et al. 1995) have been described, but little information of success rates was described.

There are several factors that influence the efficience of an SE pathway, such as: genotype, type, age, sanitary and physiological conditions of the explant-donor plant, and the external environment that include composition of media and physical culture conditions (e.g. light, temperature, pH, humidity, solid or liquid medium) (Pinto et al. 2008). Also, interactions between these factors influence induction and expression profiles and several of these parameters (genotype, year of sampling, basal medium and plant growth regulators) were analyzed here for this *Pinus* hybrid, throughout the whole somatic embryogenesis process up to the acclimatized embling.

Basal medium and PGR during Initiation and Proliferation

Several initiation media were tested (ES1-ES6; Table 1) with mDCR and mLV as basal formulations, supplemented with combinations of BAP and 2,4-D or CPPU. EM induction was obtained in all tested media, but the ES5 medium presented the highest mean initiation rate (21.44% in 2013), and was the only medium where it was possible to establish ECLs from all OP families.

Litvay medium was developed initially for conifer cell suspensions, mostly based on the chemical composition of the developing ovule prior to fertilization (Litvay et al. 1985). Since then, Litvay basal medium, with various modifications, has been used to initiate SE in several *Pinus* species, including *P. strobus* (Klimaszewska et al. 2001; Park et al. 2006), *P. pinaster* (Lelu-Walter et al. 2006; Park et al. 2006; Humánez et al. 2012), *P. banksiana, P. sylvestris* (Park et al. 2006), *P. pinea* (Carneros et al. 2009) and *P. radiata* (Hargreaves et al. 2009). For the hybrid in study, a modified mLV proved to be the best induction medium, supporting the general adequacy of this basal medium in *Pinus* genotypes. DCR medium, which was also tested here, has been already used to induce embryogenic cultures from *P.elliottii* by Newton et al. (1995), but with an extremely low initation rate (9%). David et al. (1995) used a modified DCR to induce somatic embryogenesis of *P. caribaea*, but no information of initiation rates was provided by the authors. When compared with DCR medium, mLV medium showed superior results in SE initiation of at least four of the mentioned *Pinus* species, *P. banksiana, P. sylvestris, P. strobus* and *P. pinaster* (Park et al. 2006; Humánez et al. 2012), which support our data.

Although our results show benefits of using of mLV, the PGR combination had a stronger influence on initiation and establishment rates than basal medium composition. The combination of 2 $mg.L^{-1}$ of 2,4-D and 1 $mg.L^{-1}$ of BAP promoted the highest initiation rates, 20.13% (ES2) and 21.44% (ES5). Lelu-Water et al. (2006) predicted for *P. pinaster*, a 3.6 times higher initiation rate (using the same basal medium and BAP/2,4-D concentrations) compared with mLV medium supplemented with half of the PGR concentrations. Moreover, the highest mean frequencies of extrusion and SE initiation for *P. pinea* were also obtained on a mLV medium with similar PGR concentrations (Carneros et al. 2009). The *P. elliottii* intiation was also possible with the same PGR concentrations but using DCR medium (Newton et al. 1995).

More recently, N-(2-Chloro-4-pyridyl)-N'-phenylurea (CPPU), a relatively novel and strong cytokinin, when used alone efficiently induced SE in angiosperm species (e.g. Fiore et al. 2002). Its efficiency was also demonstrated for *P. pinaster* and *P. banksiana* (Park et al. 2006). However, in *Pinus elliottii x Pinus caribaea* CPPU had no advantage in the majority of the OP families tested, supporting that this PGR has no effect on this hybrid. Moreover, after several subcultures in proliferation CPPU-containing media (ES4 and ES6), ECLs tissues became darker, and decreased growth rates than in other media. Normal vigor in proliferating EM was recovered, when ECLs from ES4 and ES6 were subcultured on media supplemented with 2,4-D and BAP, after about four months. Similar observations were made on *P. strobus* and *P. sylvestris* EM when initiated in CPPU-containing media (Park et al. 2006).

Year of sampling for Initiation

Initition experiments were peformed in two consecutive years, using the same collection data, but the explants from the first year were the most responsive. While the initiation rates in 2013 range from 10-36%, in 2014 the rates range from 0 to 28% among families. Differences in pine *Se* initiation occurring during different years have been reported by other autors. Liao and Amerson (1995), Miguel et al. (2004) and Carneros et al. (2009) experienced an increase in initiation rates of the respective species under study in the second year of collection. For *P. oocarpa*, Lara-Chavez et al. (2011) described similar initiation rates over the two consecutive years of analysis, but only on the second one was possible to establish ECLs. On the other hand, for *P. banksiana* and *P. strobus,*

experiments made with cones collected in New Brunswick (Canada) in 2003 and 2004 showed a decrease of the initiation rates in the second year. The authors attributed this phenomenon to the unusually cold spring that was experienced in New Brunswick in 2004, leading to a delay on the zygotic embryos development (Park et al. 2006).

It is well known that the development stage of the zygotic embryo, and consequently the collection date, strongly influences the response to the induction of SE (Miguel et al. 2004; Humánez et al. 2012). In our case, and concerning the period of competence to induce the SE, a previous study of the zygotic embryo development of *P. elliottii* var. *elliottii* x *P. caribaea* var. *hondurensis* was performed with the analyzes of immature seeds collected weekly, during 3 months. The period with the majority of the zygotic embryos in the early developmental stage was identified as the most responsive to induce EM (data not shown). The differential responses over different years may also be related to different seed/embryo developmental conditions encountered on the mother trees. Furthermore, parental trees growing in the field can be exposed to variable stresses on a year-to-year basis, which could impact on explant quality and responsiveness, possibly by affecting the levels of endogenous growth regulators and other metabolites (Lara-Chavez et al. 2011).

Genotype influence

It has been described that the several phases of plant regeneration by SE are under genetic control, being the initiation phase more affected by this control, indicating that this is the phase that can be manipulated most effectively by breeding due to the existence of a large amount of genetic variation (Park 2002). Significant differences in SE response among pine families, such as *P. elliottii* (Liao & Amerson 1995), *P. taeda* (Tang et al. 2001; Pullman & Johnson 2002), *P. pinaster* (Miguel et al. 2004; Park et al. 2006), *P. pinea* (Carneros et al. 2009), *P. radiata* (Hargreaves et al. 2009), and *P. halepensis* (Montalbán et al. 2013) have been reported. In the present work we also found different performances depending on the mother tree (Figure 2), being more responsive in the families A, B and E, in contrast with the families C and D. Differences in the ability of the families to achieve initiation indicate that proper selection of mother trees, or a few cases, the use of control crosses selecting a favourable male parent, could be used to increase the number of captured genotypes (Niskanen et al. 2004; Carneros et al. 2009; Montalbán et al. 2012). For *P. taeda* the initiation rate was improved from 1.5 to 9.2-fold by switching the mother

and pollen parent in each cross (MacKay et al. 2006). The authors also suggested that the use of the megagametophyte might extend the influence of the mother tree into culture to a greater degree and thus account for the importance of maternal effects. In fact for *P. radiata* the initiation rates increased significantly with the isolation of the immature zygotic embryos prior to inoculation (Hargreaves et al. 2009; 2011).

It is notorious in this *Pinus* hybrid that the genotype had also a strong influence on the maturation capacity, among families and between ECLs within each family (Figures 5 and 6). Within the family B it was well noticed that the genotype has a significant influence on maturation, since the number of mature *Se* / gFW range from 5 to 915. Montalbán et al. (2010) on their assays to improve maturation of *P. radiata* ECLs also noted that within the same family the maturation capacity is widely variable. More recently Humanéz et al. (2012) reinforce the genotype influence in the maturation of *P. pinaster Se*, analyzing different ECLs. Also, in general the ECLs from families A, B and E achieved a higher number of mature *Se* / gFW than ECLs from the other families. Niskanen et al. (2004) evaluated the genotype effect on the maturation of *P. sylvestris* somatic embryos, concluding that the mother's genotype had a significant effect on mature somatic embryo production, but differences among lines within families was also detected.

However, depiste the differences found, a major advantage of this SE protocol is that it induced SE in all families tested.

ABA concentration and proliferation medium effects during maturation

The influence of ABA concentration on embryo maturation ability was also tested. In many coniferous species, *Se* maturation could only be achieved by supplementation of exogenous ABA to the maturation medium. ABA is known to function effectively in switching embryo developmental pathway from proliferation to maturation and prevents the developing embryos from germinating precociously (Tang et al. 2001; Montalbán et al. 2010). In pine species, concentrations of ABA ranging from 10 to 150 μM, have been used during maturation experiments (Newton 1995; Klimaszewska et al. 2001; Tang et al. 2001; Miguel et al. 2004; Lara-Chavez et al. 2011; Humánez et al. 2012; Montalbán et al. 2012). For *P. radiata* (Montalbán et al. 2010) and *P. pinaster* (Alvarez et al. 2013) were tested different ABA concentrations ranging between 40 and 120 μM on the maturation medium, and for both species no significant differences were found among treatments. In the hybrid,

although, no significant differences were observed in the mature *Se* number between the different treatments, the lowest ABA concentration seemed to be beneficial in some ECLs. Álvarez et al. (2013) demonstrated that the ABA concentration used for maritime pine EM maturation can be reduced at least to 40 μM, without a significant reduction of maturation yields or quality of embryos.

We also analyzed the effect of the proliferation medium in the maturation ability, and found that the ECLs previously subcultured in ES5 proliferation medium, produced a significantly higher number of mature *Se*. Lara-Chavez et al. (2011) also observed that *P. oocarpa* maturation ability was influenced by the proliferation medium. Our data reinforce the selection of the basal medium mLV for the various phases of somatic embryogenesis process of the hybrid *Pinus elliottii x Pinus caribaea*, and therefore the selection of the ES5 medium to EM initiation and proliferation.

Plant Conversion

The cotyledonary *Se* obtained in the second experiment (II) were successfully germinated on half strength mLV, with germination rates ranging from 70-88%, while the plantlet conversion rate varied from 60 to 86%. Newton et al. (1995) published a substantially lower germination rate, of only 8.8%, for *P. elliottii*, one of the parents of the hybrid in study. For *P. pinaster* a similar *Se* germination rate after four weeks in culture, around 78%, was recently obtained by Álvarez and co-workers (2013). An identical germination rate was also achieved for *P. pinea* by Carneros et al. (2009), but the plantlet conversion was only 30%. Regarding to plantlet conversion rates, our results are similar to the ones obtained for *P. caribaea* (David et al. 1995), *P. strobus* (Klimaszewska et al. 2001), *P. radiata* (Montalbán et al. 2010) *a*nd *P. halepensis* (Montalbán et al. 2013).

These somatic seedlings were transplanted to a potting mix in the greenhouse. After 4 months, the survival percentage of the plantlets ranged between 78 and 85%. Identical survival rates were reported for *P. pinaster* (Lelu-Walter et al. 2006; Alvarez et al. 2013) and *P. radiata* (Montalbán et al. 2010).

Genetic stability

True-to-typeness is an important requisite in cloning protocols ensuring that regenerated plants are genetically identical to donor material (Conde et al. 2004; Lopes et al. 2006;

Marum et al. 2009). In this study FCM analysis was used as diagnostic tool to analyze the stability of the SE process in this hybrid, using as source material leaves from the mother trees, EM with different ages and emblings produced by this process.

The results reported show histograms with typical diploid profiles, and average CV values ~6%. CV values are important in FCM studies, varying between 1 and 10% for plant cells, which are considered by some authors as an elementar criterion for the quality of the analysis (e.g. Loureiro et al. 2006). Values around 4% were obtained for *Juniperus* micropropagated plants (Loureiro et al. 2007a), and lower CV for EM of *Pinus pinaster* (Marum et al. 2009), but presence of cytosolic compounds, or other factors such as pH may lead to higher CV values which is particularly frequent in woody species (e.g. Loureiro et al. 2006).

All the mother samples analyzed have the same ploidy level. Also we demonstrated that our SE protocol induced no ploidy changes in the EM samples (3 and 15 months old), although SE-derived tissues are pointed out in literature as potentially instable. It was observed similar DNA content values (pg/2C) between OP families trees and the respective emblings, presenting DNA contents of ~46-47 pg/2C, though, emblings had in general higher DNA content values than mother plants and lower than SE masses. The values of DNA content are strongly influenced by cytosolic compounds that interfere with *Pi*-DNA interaction (Noirot et al. 2000; Loureiro et al. 2006). DNA content differences found between the tissues tested (lower in leaves of the mother trees) may suggest that, as proposed for other adult woody species, adult tissues contain higher levels of compounds that may affect the analysis, giving DNA values slightly different from more juvenile material. In fact, *Pinus* spp. have high percentage of secondary metabolites, such as total polyphenols, condensed tannins, and free and esterified phenolic acids (Marum et al. 2009). These cytosolic componds were found to interfere on the FCM analysis of other woody species like e.g. *Coffea liberica var. Dewevrei* (Noirot et al. 2000), *Olea europaea* (Brito et al. 2008) or *Vitis vinifera* (Leal et al. 2006; Prado et al. 2010). This interference is due to the fact that chromatin is exposed to cytosolic compounds during nuclear isolation. As the nuclei are stained by PI within a crude homogenate, the staining is influenced not only by the composition of the nuclear isolation buffer, but also by the compounds present in the cytosol (Doležel & Bartoš 2005). Supporting this hypothesis Noirot et al. (2000) and Loureiro et al. (2006) stressed that cytosolic compounds can promote changes on nuclear

DNA content estimates by up to 20%. Maximum differences (~8%) were observed between the mother tree A and the emblings clones A.36, therefore clearly fitting within this range of variation. Our data suggest that EM (juvenile material) had lower cytosolic levels than emblings and than needles from the mother plant. As material ages, the DNA content values decrease to values closer to those of mother trees.

FCM was also used to evaluate the genetic variability of somatic embryogenesis-derived tissues in *Pinus pinaster*, which showed DNA content values similar to those of zygotic embryos (Marum et al. 2009). To our knowledge the DNA content of the tropical hybrid in study was previously determined only by Williams et al. (2002) using megagametophytes (highly juvenile material) as source material. Although it is assumed that the comparison of the data obtained in different laboratories may have some constraints due to the use of different reference standards (Doležel and Bartoš 2005), we verified that the values published by Williams et al. (2002) (47.74 and 48.22 pg/2C) are close to the values obtained by us for the EM (47.84 to 51.12 pg/2C) and emblings (46.26 to 49.36 pg/2C).

Conclusion

This is the first report that describes a simple protocol that represents a crucial kick-off for large-scale *Se* production of *P. elliottii x P. caribaea*, a *Pinus* hybrid with major economic value. We assessed relevant variables in the different stages of the SE process, including: intiation, proliferation, maturation, conversion to plants and acclimatization. We conclude that mLV is the most appropriate basal medium for the SE process, and adequate PGR combinations and concentration were selected for the different SE phases. This SE process also demonstrated that it does not promote major genetic changes, and emblings showed by FCM true to typeness. Taking into account the data obtained in this work for maturation, conversion and survival of the emblings, we believe that this protocol will be very useful for the propagation of selected families for implementation of multi-varietal forestry (MVF), simplifying breeding programs of interspecific *Pinus* hybrids.

References

Alvarez, José M, Natalia Bueno, Millán Cortizo, and Ricardo J Ordás. 2013. "Improving Plantlet Yield in *Pinus pinaster* Somatic Embryogenesis." *Scandinavian Journal of Forest Research* 28 (7): 613–20.

Arnold, S von, and I Hakman. 1988. "Plantlet Regeneration in Vitro via Adventitious Buds and Somatic Embryos in Norway Spruce (*Picea abies*)." In *Genetic Manipulation of Woody Plants*, edited by JW Hanover, DE Keathley, CM Wilson, and G Kuny, 199–215. Springer US. doi:10.1007/978-1-4613-1661-9_12.

Bonga, J M. 2015. "A Comparative Evaluation of the Application of Somatic Embryogenesis, Rooting of Cuttings, and Organogenesis of Conifers." *Canadian Journal of Forest Research* 45: 379–83. doi:379-383, 10.1139/cjfr-2014-0360.

Brito, Gina, João Loureiro, Tina Lopes, Eleazar Rodriguez, and Conceição Santos. 2008. "Genetic Characterisation of Olive Trees from Madeira Archipelago Using Flow Cytometry and Microsatellite Markers." *Genetic Resources and Crop Evolution* 55 (5): 657–64. doi:10.1007/s10722-007-9268-8.

Campbell, Malcolm M, Amy M Brunner, Helen M Jones, and Steve H Strauss. 2003. "Forestry's Fertile Crescent: The Application of Biotechnology to Forest Trees." *Plant Biotechnology Journal* 1 (3): 141–54.

Carneros, E, C Celestino, K Klimaszewska, Y-S Park, M Toribio, and J M Bonga. 2009. "Plant Regeneration in Stone Pine (*Pinus pinea* L.) by Somatic Embryogenesis." *Plant Cell, Tissue and Organ Culture (PCTOC)* 98 (2): 165–78.

Conde, P, J Loureiro, and C Santos. 2004. "Somatic Embryogenesis and Plant Regeneration from Leaves of *Ulmus minor* Mill." *Plant Cell Reports* 22 (9): 632–39. doi:10.1007/s00299-003-0735-1.

Conde, Paula, Alexandra Sousa, Armando Costa, and Conceição Santos. 2008. "A Protocol for *Ulmus minor* Mill. Micropropagation and Acclimatization." *Plant Cell, Tissue and Organ Culture* 92 (1): 113–19. doi:10.1007/s11240-007-9310-8.

Cyr, David R, and Krystyna Klimaszewska. 2002. "Conifer Somatic Embryogenesis: II. Applications." *Dendrobiology* 48: 41–49.

David, A, E Laine, and H David. 1995. "Somatic Embryogenesis in *Pinus caribaea*." In *Somatic Embryogenesis in Woody Plants*, 145–81. Springer.

Doležel, Jaroslav, and J A N Bartoš. 2005. "Plant DNA Flow Cytometry and Estimation of Nuclear Genome Size." *Annals of Botany* 95 (1): 99–110.

Fernandes, P., E. Rodriguez, G. Pinto, I. Roldan-Ruiz, M. De Loose, and C. Santos. 2008. "Cryopreservation of *Quercus suber* Somatic Embryos by Encapsulation-Dehydration and Evaluation of Genetic Stability." *Tree Physiology* 28 (12): 1841–50. doi:10.1093/treephys/28.12.1841.

Fiore, Stefania, Fabio De Pasquale, Francesco Carimi, and Maurizio Sajeva. 2002. "Effect of 2, 4-D and 4-CPPU on Somatic Embryogenesis from Stigma and Style Transverse Thin Cell Layers of Citrus." *Plant Cell, Tissue and Organ Culture* 68 (1): 57–63.

Ford, C S, L J Fischer, N B Jones, S A Nigro, N P Makunga, and J van Staden. 2005. "Somatic Embryogenesis in *Pinus patula*." In *Protocol for Somatic Embryogenesis in Woody Plants*, 121–39. Springer.

Gupta, P K, and D J Durzan. 1985. "Shoot Multiplication from Mature Trees of Douglas-Fir

(*Pseudotsuga menziesii*) and Sugar Pine (*Pinus lambertiana*).” *Plant Cell Reports* 4 (4): 177–79. doi:10.1007/BF00269282.

Hargreaves, C. L., C. B. Reeves, K. I. Find, K. Gough, M. I. Menzies, C. B. Low, and T. J. Mullin. 2011. “Overcoming the Challenges of Family and Genotype Representation and Early Cell Line Proliferation in Somatic Embryogenesis from Control-Pollinated Seeds of *Pinus radiata*.” *New Zealand Journal of Forestry Science* 41 (January): 97–114.

Hargreaves, Cathy L., Cathie B. Reeves, Jens I. Find, Keiko Gough, Puthiyaparambil Josekutty, Dolina B. Skudder, Susan A. van der Maas, et al. 2009. “Improving Initiation, Genotype Capture, and Family Representation in Somatic Embryogenesis of *Pinus radiata* by a Combination of Zygotic Embryo Maturity, Media, and Explant Preparation.” *Canadian Journal of Forest Research* 39 (8): 1566–74. doi:10.1139/X09-082.

Humánez, A., M. Blasco, C. Brisa, J. Segura, and I. Arrillaga. 2012. “Somatic Embryogenesis from Different Tissues of Spanish Populations of Maritime Pine.” *Plant Cell, Tissue and Organ Culture (PCTOC)* 111 (3): 373–83. doi:10.1007/s11240-012-0203-0.

Kim, YW, and HK Moon. 2007. “Regeneration of Plant by Somatic Embryogenesis in *Pinus rigida*× *P. taeda*.” *In Vitro Cellular & Developmental Biology-Plant* 43 (4): 335–42. doi:10.1007/s116727-007-9045-6.

Klimaszewska, Krystyna, Yill-Sung Park, Cathy Overton, Ian Maceacheron, and Jan M. Bonga. 2001. “Optimized Somatic Embryogenesis in *Pinus strobus* L.” *In Vitro Cellular & Developmental Biology - Plant* 37 (3): 392–99. doi:10.1007/s11627-001-0069-z.

Lara-Chavez, Alejandra, Barry S Flinn, and Ulrika Egertsdotter. 2011. “Initiation of Somatic Embryogenesis from Immature Zygotic Embryos of Oocarpa Pine (*Pinus oocarpa* Schiede Ex Schlectendal).” *Tree Physiology* 31 (5): 539–54. doi:10.1093/treephys/tpr040.

Leal, F, J Loureiro, E Rodriguez, M S Pais, C Santos, and O Pinto-Carnide. 2006. “Nuclear DNA Content of *Vitis vinifera* Cultivars and Ploidy Level Analyzes of Somatic Embryo-Derived Plants Obtained from Anther Culture.” *Plant Cell Reports* 25 (9): 978–85. doi:10.1007/s00299-006-0162-1.

Lelu-Walter, MA, M Bernier-Cardou, and K Klimaszewska. 2006. “Simplified and Improved Somatic Embryogenesis for Clonal Propagation of *Pinus pinaster* (Ait.).” *Plant Cell Reports* 25 (8): 767–76. doi:10.1007/s00299-006-0115-8.

Lelu-Walter, MA, and LE Pâques. 2009. “Simplified and Improved Somatic Embryogenesis of Hybrid Larches (*Larix X Eurolepis* and *Larix X Marschlinsii*). Perspectives for Breeding.” *Annals of Forest Science* 66 (1): 104. doi:10.1051/forest/2008079.

Liao, Y. K., and H. V. Amerson. 1995. “Slash Pine (*Pinus elliottii* Engelm.) Somatic Embryogenesis I. Initiation of Embryogenic Cultures from Immature Zygotic Embryos.” *New Forests* 10 (2). Kluwer Academic Publishers: 145–63. doi:10.1007/BF00033404.

Litvay, J D, D C Verma, and M A Johnson. 1985. “Influence of a Loblolly Pine (*Pinus taeda* L.). Culture Medium and Its Components on Growth and Somatic Embryogenesis of the Wild Carrot (*Daucus carota* L.).” *Plant Cell Reports* 4 (6): 325–28. doi:10.1007/BF00269890.

Lopes, T, G Pinto, J Loureiro, A Costa, and C Santos. 2006. “Determination of Genetic Stability in Long-Term Somatic Embryogenic Cultures and Derived Plantlets of Cork Oak Using Microsatellite Markers.” *Tree Physiology* 26: 1145–52.

Loureiro, J, E Rodriguez, J Dolezel, and C Santos. 2006. “Flow Cytometric and Microscopic Analysis of the Effect of Tannic Acid on Plant Nuclei and Estimation of DNA Content.” *Annals of Botany* 98 (3): 515–27. doi:10.1093/aob/mcl140.

Loureiro, J., A. Capelo, G. Brito, E. Rodriguez, S. Silva, G. Pinto, and C. Santos. 2007a. "Micropropagation of *Juniperus phoenicea* from Adult Plant Explants and Analysis of Ploidy Stability Using Flow Cytometry." *Biologia Plantarum* 51 (1): 7–14. doi:10.1007/s10535-007-0003-2.

Loureiro, João, Eleazar Rodriguez, Jaroslav Dolizel, and Conceição Santos. 2007b. "Two New Nuclear Isolation Buffers for Plant DNA Flow Cytometry: A Test with 37 Species." *Annals of Botany* 100: 875–888. doi:10.1093/annbot/mcm152.

MacKay, J. J., M. R. Becwar, Y. -S. Park, J. P. Corderro, and G. S. Pullman. 2006. "Genetic Control of Somatic Embryogenesis Initiation in Loblolly Pine and Implications for Breeding." *Tree Genetics & Genomes* 2 (1): 1–9. doi:10.1007/s11295-005-0020-2.

Marum, Liliana, Catarina Estêvão, M. Margarida Oliveira, Sara Amâncio, Lucília Rodrigues, and Célia Miguel. 2004. "Recovery of Cryopreserved Embryogenic Cultures of Maritime Pine - Effect of Cryoprotectant and Suspension Density." *Cryoletters* 25 (5). Cryoletters: 363–74.

Marum, Liliana, João Loureiro, Eleazar Rodriguez, Conceição Santos, M. Margarida Oliveira, and Célia Miguel. 2009. "Flow Cytometric and Morphological Analyzes of *Pinus pinaster* Somatic Embryogenesis." *Journal of Biotechnology* 143 (4): 288–95. doi:10.1016/j.jbiotec.2009.08.001.

Miguel, Célia, Sónia Gonçalves, Susana Tereso, Liliana Marum, João Maroco, and M. Margarida Oliveira. 2004. "Somatic Embryogenesis from 20 Open-Pollinated Families of Portuguese Plus Trees of Maritime Pine." *Plant Cell, Tissue and Organ Culture* 76 (2): 121–30. doi:10.1023/B:TICU.0000007253.91771.e3.

Montalbán, I. A., A. Setién-Olarra, C. L. Hargreaves, and P. Moncaleán. 2013. "Somatic Embryogenesis in *Pinus halepensis* Mill.: An Important Ecological Species from the Mediterranean Forest." *Trees* 27 (5): 1339–51. doi:10.1007/s00468-013-0882-0.

Montalbán, I.A., N De Diego, and P Moncaleán. 2012. "Enhancing Initiation and Proliferation in Radiata Pine (*Pinus radiata* D. Don) Somatic Embryogenesis through Seed Family Screening, Zygotic Embryo Staging and Media." *Acta Physiologiae Plantarum* 34 (2): 451–60. doi:10.1007/s11738-011-0841-6.

Montalbán, IA, N De Diego, and P Moncaleán. 2010. "Bottlenecks in *Pinus radiata* Somatic Embryogenesis: Improving Maturation and Germination." *Trees* 24 (6): 1061–71. doi:10.1007/s00468-010-0477-y.

Montalbán, IA, N De Diego, and P. Moncaleán. 2011. "Recent Observations Regarding *Pinus radiata* Somatic Embryogenesis Using Juvenile and Mature Material." In *Proceedings of the IUFRO Working Party 2.09.02*, 11–20. Suwon, Republic of Korea.

Murashige, T, and F Skoog. 1962. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures." *Physiologia Plantarum* 15 (3): 473–97. doi:10.1111/j.1399-3054.1962.tb08052.x.

Murthy, BNS, and PK Saxena. 1994. "Somatic Embryogenesis in Peanut (*Arachis hypogaea* L.): Stimulation of Direct Differentiation of Somatic Embryos by Forchlorfenuron (CPPU)." *Plant Cell Reports* 14 (2): 145–50. doi:10.1007/BF00233779.

Newton, RJ, KA Marek-Swize, ME Magallanes-Cedeno, N Dong, S Sen, and SM Jain. 1995. "Somatic Embryogenesis in Slash Pine (*Pinus elliottii* Engelm.)." In *Somatic Embryogenesis in Woody Plants, Volume 3 - Gymnosperms*, edited by SM Jain, PK Gupta, and RJ Newton, 183–95. doi:10.1007/978-94-011-0960-4_11.

Nikles, DG. 2000. "Experience with Some *Pinus* Hybrids in Queensland, Australia." In

Proceedings of QFRI/CRC-SPF Symposium: Hybrid Breeding and Genetics of Forest Trees, edited by HS Dungey, MJ Dieters, and DG Nikles, 27–43. Noosa, Queensland, Australia: Department of Primary Industries, Brisbane.

Niskanen, AM, J Lu, S Seitz, K Keinomen, K von Weissenberg, and A Pappinen. 2004. "Effect of Parent Genotype on Somatic Embryogenesis in Scots Pine (*Pinus sylvestris*)." *Tree Physiol* 24 (11): 1259–65. doi:10.1093/treephys/24.11.1259.

Noirot, M, P Barre, J Louarn, C Duperray, and S Hamon. 2000. "Nucleus–cytosol Interactions—a Source of Stoichiometric Error in Flow Cytometric Estimation of Nuclear DNA Content in Plants." *Annals of Botany* 86: 309–16. doi:10.1006/anbo.2000.1187.

Park, Y. S., M. A. Lelu-Walter, L. Harvengt, J. F. Trontin, I. MacEacheron, K. Klimaszewska, and J. M. Bonga. 2006. "Initiation of Somatic Embryogenesis in *Pinus banksiana, P. strobus, P. pinaster*, and *P. sylvestris* at Three Laboratories in Canada and France." *Plant Cell, Tissue and Organ Culture* 86 (1): 87–101. doi:10.1007/s11240-006-9101-7.

Park, Yill-Sung. 2002. "Implementation of Conifer Somatic Embryogenesis in Clonal Forestry: Technical Requirements and Deployment Considerations." *Annals of Forest Science* 59 (5-6): 651–56. doi:10.1051/forest:2002051.

Park, YS, and J Bonga. 2011. "Applications of Somatic Embryogenesis in Forest Management and Research." In *Proceedings of the IUFRO Working Party 2.09.02*, 3–9.

Pinto, G, S Silva, Y Park, L Neves, C Araújo, and C Santos. 2008. "Factors Influencing Somatic Embryogenesis Induction in *Eucalyptus globulus* Labill.: Basal Medium and Anti-Browning Agents." *Plant Cell, Tissue and Organ Culture* 95 (1): 79–88. doi:10.1007/s11240-008-9418-5.

Pinto, G, H Valentim, A Costa, S Castro, and C Santos. 2002. "Somatic Embryogenesis in Leaf Callus from a Mature *Quercus suber* L. Tree." *In Vitro Cellular & Developmental Biology - Plant* 38 (6): 569–72. doi:10.1079/IVP2002352.

Prado, María Jesús, Eleazar Rodriguez, Laura Rey, María Victoria González, Conceição Santos, and Manuel Rey. 2010. "Detection of Somaclonal Variants in Somatic Embryogenesis-Regenerated Plants of *Vitis vinifera* by Flow Cytometry and Microsatellite Markers." *Plant Cell, Tissue and Organ Culture (PCTOC)* 103 (1): 49–59. doi:10.1007/s11240-010-9753-1.

Pullman, Gerald S., and Shannon Johnson. 2002. "Somatic Embryogenesis in Loblolly Pine (*Pinus taeda* L.): Improving Culture Initiation Rates." *Annals of Forest Science* 59 (5-6). EDP Sciences: 663–68. doi:10.1051/forest:2002053.

Pullman, GS, J Mein, S Johnson, and Y Zhang. 2005. "Gibberellin Inhibitors Improve Embryogenic Tissue Initiation in Conifers." *Plant Cell Reports* 23 (9): 596–605. doi:10.1007/s00299-004-0880-1.

Salajova, T, and J Salaj. 2005. "Somatic Embryogenesis in *Pinus nigra*: Embryogenic Tissue Initiation, Maturation and Regeneration Ability of Established Cell Lines." *Biologia Plantarum* 49 (3): 333–39. doi:10.1007/s10535-005-0003-z.

Shepherd, M, M Cross, MJ Dieters, and R Henry. 2002. "Branch Architecture QTL for *Pinus elliottii* var. *elliottii X Pinus caribae*a var. *hondurensis* Hybrids." *Annals of Forest Science* 59 (5-6): 617–25. doi:10.1051/forest:2002047.

Stojičić, Dragana, Branka Uzelac, Dušica Janošević, Ljubinka Ćulafić, and Snežana Budimir. 2007. "Induction of Somatic Embryogenesis in *Pinus heldreichii* Culture." *Archives of Biological Sciences* 59 (3): 199–202. doi:10.2298/ABS0703199S.

Tang, W, Z Guo, and F Ouyang. 2001. "Plant Regeneration from Embryogenic Cultures Initiated

from Mature Loblolly Pine Zygotic Embryos." *In Vitro Cellular & Developmental Biology-Plant* 37 (5): 558–63. doi:10.1007/s11627-001-0097-8.

CHAPTER IV

CRYOPRESERVATION OF EMBRYOGENIC CULTURES OF THE HYBRID *PINUS ELLIOTTII* VAR. *ELLIOTTII X PINUS CARIBAEA* VAR. *HONDURENSIS AND GENETIC STABILITY*

Chapter prepared to be submitted as an original paper to a SCI journal:

Nunes S, Marum L, Pereira VT, Almeida T, Farinha N, Dias MC, Santos C. 2016. Cryopreservation of embryogenic cultures of the hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis and genetic stability*

Abstract

Clonal breeding programs of the highly important *Pinus* interspecific hybrid *Pinus elliottii var. elliottii* and *Pinus caribaea var. hondurensis* require the establishment of robust cryopreservation protocols. Embryogenic masses (EM) of this hybrid were cryopreserved by a slow-freezing method. The effects of DMSO based cryoprotectant solutions, sugar cryoprotectants such as sucrose or maltose (each reaching 0.4M) as well as different times of pretreatments and pre-cooling storage have been tested. Our results show that the addition of DMSO in a mixture of PEG 4000 and sucrose (PSD solution), instead of DMSO alone was beneficial for recovery of cryopreserved cultures. This parameter together with the time of pre-cooling storage before plunging in liquid nitrogen, were the factors that most influenced the survival and regrowth rates of embryonal masses. A pretreatment combination of sucrose (0.4M) and 5% PSD followed by a pre-cooling storage of 24 hours allowed the cryopreservation and regrowth of embryogenic cell lines without major genetic variations or loss of embryogenic potential. The genotype and treatment conditions clearly influenced the response, but all nine tested embryogenic cell lines survived to the cryopreservation procedures. Somatic embryos maturation and conversion of recovered EM took place according to our standard protocol, using mLV medium.

Keywords: *Pinus elliottii x Pinus caribaea*, somatic embryogenesis, cryopreservation, cryoprotectant, pre-cooling storage, genetic stability.

Introduction

Due to the enormous industrial importance of the hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis*, increasing pressure is put on developing efficient breeding programs allowing the cloning and long term preservation of elite individuals. In fact this hybrid has improved characteristics when compared to its parents, apparently derived from a complementary recombination of traits like growth rate, including superior branch quality and more uniform wood from *P. caribaea*, combined with high wood density, stem straightness, wind-firmness and adaptability to wet sites of *P. elliottii* (Nikles 2000; Dieters & Brawner 2007). Biotechnological approaches of cloning elite individuals are being developed including the establishment of somatic embryogenesis (SE) protocols for forest species (e.g. Pinto et al. 2002; 2008; Marum et al. 2009). In the previous work, we developed a SE protocol that proved to be efficient with different families tested of this hybrid (Chapter III of this thesis), using immature zygotic embryos as starting explants. However, the industrial use of SE also requires a full cryopreservation/recovery protocol of embyogenic masses, which has not yet been developed for this interspecific hybrid.

Cryopreservation allows the storage of biological material at ultra-low temperature of liquid nitrogen (–196°C), and is the only method currently available to ensure the safe and cost-effective long-term conservation of genetic resources of species that have recalcitrant seeds or are vegetatively propagated, including apical or axillary buds, pollen, somatic embryos, and embryogenic tissues (Maeno & Hawtin 2000; Engelmann 2004; Fernandes et al. 2008; Martinez-Montero & Harding 2015). Therefore, SE in conjugation with cryopreservation are two essential tools for the implementation of multi-varietal forestry (MVF). Through the cryo-storage it is possible to preserve a high number of genotypes, preserving also their juvenile characteristics, until getting the progeny tests results. Cryopreservation also prevents the loss of embryogenic capacity and the occurrence of putative somaclonal variations frequently found in long-term subcultures. It also reduces costs associated to the regular subculturing, and prevents the risk of losing plant material due to contaminations, technical or human errors (Ford et al. 2000b; Reinhoud et al. 2000; von Arnold et al. 2002).

From the different procedures that have been used for cryopreservation of plant cells, slow freezing method or two-step freezing, vitrification, encapsulation-dehydration,

encapsulation-vitrification, desiccation and droplet-vitrification (Reinhoud et al. 2000; Engelmann 2011) the first one is the most used to cryopreserved conifer embryogenic cultures. Embryogenic cells are water-rich cells and highly susceptible to the ice crystals formed at low temperatures used during cryopreservation. They are also sensitive to the dessication procedures that some cryopreservation protocols require. Different cryoprotectant solutions are used to lower the freezing-point in plant cells, resulting in avoidance of crystallization and maintaining a minimal moisture level so allowing cell viability. The most used cryoprotectants are sugars, sugar-alcohols and DMSO (dimethyl sulfoxide) (Reinhoud et al. 2000; Marum et al. 2004). Also, cryo-storage of embryogenic cells requires optimization as excessive dehydration may lead to damaging events associated to e.g., concentration of intracellular salts and changes in the cell membrane permeability (Engelmann 2000).

The first protocol for cryopreservation of conifer somatic embryogenic cultures was described for *Picea glauca* by Kartha et al. (1988), a slow freezing procedure. Since then several reports successfully used this method in embryogenic cell lines (ECLs) of *P. caribaea* (Laine et al. 1992; David et al. 1995), *P. taeda* (Gupta et al. 1987), *P. patula* (Ford et al. 2000a,b), *P. radiata* (Hargreaves et al. 2002), *P. pinaster* (Marum et al. 2004; Álvarez et al. 2012), *P. sylvestris* (Häggman et al. 1998; Latutrie & Aronen 2013), *P. roxburghii* (Mathur et al. 2003; Malabadi & Nataraja 2006) and *P. nigra* (Salaj et al. 2007;2011). However, to our knowledge no cryopreservation protocol (including recovery and plant regeneration) has been described for the economically relevant interspecific hybrid *P. elliottii* var. *elliottii x P. caribaea* var. *hondurensis*, nor its parent *P. elliottii*.

As described above, cryopreservation exposes plant material to physical, chemical, and physiological stresses that cause cryoinjury (Harding 2004). These stresses may affect the plant material genetic stability, since the formation of free radicals can cause, among other injuries, mutations in DNA (Dumet & Benson 2000). So, before cryopreservation can be used as a tool in biotechnology or as a conservation strategy, it is essential to verify that the cryopreservation protocol developed does not induce somaclonal variation in plants regenerated from embryogenic masses (EM). Despite its relevance, only few work has been made for the evaluation of plant material genetic stability after cryopreservation, namely for *Abies cephalonica* (Aronen et al. 1999), *Picea glauca* (DeVerno et al. 1999), *Pinus sylvestris* (Häggman et al. 1998) and more recently in *Pinus nigra* (Salaj et al. 2011).

The main objective of this work was to establish an efficient cryopreservation procedure for long-term storing embryogenic cultures of the hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis*. The effect of several cryopreservation parameters on the recovery of different cryopreserved ECLs was performed. Additionally, the maintenance of the maturation and conversion potencials of recovered ECLs was demonstrated as well as the genetic stability of the regenerated ECLs was analyzed.

Materials and Methods

Plant Material

Embryogenic cell lines (ECLs) of the five open pollinated (OP) plus mother trees of the hybrid *P. elliotti* var. *elliotti x P. caribaea* var. *hondurensis* were initiated by innoculating megagametophytes on the basal medium mLV supplemented with BAP (1 $mg.L^{-1}$) and 2,4-D (2 $mg.L^{-1}$), as described in Chapter III of this thesis. Established ECLs were maintained in the dark at 23 ± 2 ºC on proliferation medium consisting of (1) modified DCR medium (mDCR) based on DCR macro and microelements (Gupta & Durzan 1985) and MS vitamins (Murashige & Skoog 1962) supplemented with 1 $mg.L^{-1}$ 6-benzylaminopurine (BAP), 2 $mg.L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), casein hydrolysate (500 $mg.L^{-1}$), L-glutamine (250 $mg.L^{-1}$) and 100 $mg.L^{-1}$ inositol (ES2) or (2) modified Litvay's (Litvay et al. 1985) medium (mLV) with half-strength macroelements and full-strength microelements and Fe-EDTA, as described by Klimaszewska et al. (2001) supplemented with the same concentrations of plant growth regulators (PGR), casein hydrolysate (1000 $mg.L^{-1}$) and L-glutamine (500 $mg.L^{-1}$) (ES5). Both media were supplemented with 2% (w/v) sucrose and solidified with 0.4% (w/v) Gelrite (Duchefa). Subculture of the ECLs was performed every 2 weeks, for 4 to 12 months, before the beginning of the cryopreservation experiments.

Cryopreservation

Standard protocol

a) Pretreatments before freezing:

The standard cryopreservation protocol used in these experiments was based on the method described by Nörgaard et al. (1993) with minor modifications. Embryogenic suspensions were obtained by using 2 g of embryogenic masses (EMs), suspended in 20 ml of PGR-free proliferation medium in 50 ml flasks. The EMs were disaggregated with a transfer pipette and the flasks shaken to break up the clumps of tissue into a fine suspension. Samples were pretreated with sucrose as cryoprotective carbohydrate for 48 hours, according to Marum et al. (2004). On the third day, the flasks containing the embryogenic suspensions were transferred to ice and, after 15 min, DMSO was carefully added to a final concentration of 5% (v/v). After the addition of the cryoprotectant the suspensions were incubated for one hour on ice, in constant agitation. The cells were allowed to sediment in the flask (~10 min) and part of the supernatant was removed to achieve a final suspension density of 200 mg/mL. Aliquots of 1.8 mL of the pretreated embryogenic suspension were then dispensed into cryovials and were placed on a cell freezer container (Coolcell by BioCision) kept at -80ºC for 24 hours before they were plunged directly into liquid nitrogen (-196ºC).

b) *Rewarming and regrowth:*

To regrowth of cryopreserved ECLs, samples were removed from the liquid nitrogen and immediately warmed in a 45ºC water-bath until completely thawed and then transferred onto ice. The cryotubes were then surface sterilized with 70% ethanol and allowed to dry in a laminar flow hood. The content of the vials was then poured on a disk of sterile filter paper (55 mm Whatman Nº2) previously placed on sterile paper towels to drain excess of liquid. Drained filter paper was then placed on proliferation medium. Thereafter, the thawed embryogenic tissues on filter papers were subcultured every 2 weeks by transfer to fresh proliferation medium, during two months (1^{st} to 4^{th} subculture).

Effect of pre-cooling storage

In order to test the effect of pre-cooling storage in our EM, the samples were kept at -80ºC for 4 and 24 hours in the freezer container (Collcell), before they were plunged directly into liquid nitrogen. The cryopreservation and regrowth procedures were conducted as described above, with sucrose and DMSO (5%) as cryoprotectants. Three different ECLs were used for this experiment with four replicates, per genotype and condition.

Effect of DMSO based cryoprotectants

In this experiment the standard protocol was compared with the same protocol replacing the cryoprotectant DMSO by PSD (20% polyethylene glycol (PEG 4000), 20% sucrose and 20% DMSO (v/v)- FS) at the same final concentration (5%). In both treatments 4 hours were used as pre-cooling storage period, at -80ºC. Four different ECLs were used during the experiment, with four replicates per genotype condition.

Effect of carbohydrate preculture

In addition to sucrose used on the standard cryopreservation procedure described above, maltose was also tested as cryoprotective carbohydrate. Same final concentrations of maltose were used during the pretreatments. A 5% PSD solution was used as cryoprotectant and 24 hours as pre-cooling storage period at -80ºC, on five ECLs (four replicates per genotype and condition).

Comparison of cryopreservation procedures

Different pretreatment procedures were compared, the classical slow freezing method (CSF), which consists on the methodology described as standard protocol, and a short slow freezing method (SSF) based on the methodology described by Alvaréz et al. (2012). For both procedures, sucrose and 5% PSD were used as cryoprotectants. For the SSF the embryogenic cell suspension was obtained by mixing 3.16 g of actively growing EM in 12.8 mL of liquid PGR-free proliferation medium. Then, samples were pretreated with sucrose as cryoprotective carbohydrate for 24 hours. On the second day the suspensions were transferred to ice for 10 minutes without agitation and then 3.95 mL of the supernatant was removed and replaced by PSD solution (20% PEG 4000, 20% sucrose and

20% DMSO (v/v)- FS), added dropwise, to a final concentration of 5% for each component. Then the cell suspensions were incubated for one hour on ice in constant agitation. Aliquots of 1.8 mL of the pretreated embryogenic suspension were then dispensed into cryovials and were placed on the frezeer container (Collcell) kept at -80ºC for four (SSF I) or 24 hours (SSF II), before they were plunged directly into liquid nitrogen (-196ºC). Five different ECLs were used in the experiment, with four replicates per genotype and condition.

Embryogenic competence evaluation

The presence of embryogenic structures on the cryopreserved and thawed cultures was monitored by light microscopy observations of tissue samples from different ECLs, which were gently squashed in 2% acetocarmine.

Evaluation of maturation and germination capacity

Eight ECLs known to have maturation capacity and cryopreserved according to the improved CSF protocol, using sucrose and 5% PSD as cryoprotectans, were thawed and recovered from cryopreservation one month after freezing, and 15 months after the initiation experiments. The recovered samples were subjected to maturation to evaluate their ability to produce mature somatic embryos (*Se*). Non-cryopreserved samples from the same ECLs were also subjected to maturation and the results compared with the first maturation experiment made with these three months, after initiation. For the maturation experiment, 200 mg of cryo and non-cryopreserved EM, 7 days after subculture, were placed into a sterile falcon tube and re-suspended in 3 mL of liquid PGR-free proliferation medium. Then 1 mL of suspension was spread on a filter paper (Whatman nº2, 55mm) previously placed on sterile paper towels to drain excess liquid. Lastly the filter papers were placed on the surface of the maturation medium consisting of (1) mDCR basal medium supplemented with casein hydrolysate (500 $mg.L^{-1}$), L-glutamine (250 $mg.L^{-1}$) and 100 $mg.L^{-1}$ inositol or (2) mLV basal medium supplemented with casein hydrolysate (1000 $mg.L^{-1}$), L-glutamine (500 $mg.L^{-1}$). For both maturation media 80 μM of abscisic acid (ABA) and 6% (w/v) sucrose were added and solidified with 0.9% (w/v) Gelrite

(Duchefa). Three to five independent replicates were incubated per condition for 10 weeks, in the dark and in a growth chamber, at 23 ± 2 °C.

The mature *Se* were converted to plantlets after the maturation experiments, by transferring them to half-strength basal medium (mDCR ou mLV), devoid of PGR, with 0.025% activated charcoal, 2% sucrose and solidified with 0.6% Gelrite. The *Se* were maintained one week in the dark, another in subdued light and finally 3 weeks in normal light in a growth chamber, at 23 ± 2 °C, for a 16/8-h (day/night) photoperiod, under a photosynthetic photon flux density (PPFD) of approx. 50 ± 10 μmol m^{-2} s^{-1}. Emblings were then transferred to flasks with same germination medium but devoid of activated carbon being maintained in the same conditions of light and temperature.

Genetic stability by flow cytometry

Samples from cryo and non-cryopreserved embryogenic masses under proliferation were prepared to obtain nuclear suspensions. For that, small portions (~50 mg) EM were chopped in Woody Plant Buffer (WPB) (0.2 M Tris–HCl, 4 mM $MgCl_2$, 2 mM EDTA Na_2, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7.5) (e.g. Loureiro et al. 2007b). Samples were filtered through a 50 μm nylon filter. To the nuclear suspension 50 μg/ml of both propidium iodide (*Pi*, Fluka) and RNase (Sigma) were added, to stain DNA and degrade ds-RNA, respectively (Loureiro et al. 2007a,b). After incubating for 5 min at ~4ºC, samples were analyzed in an Attune® Acoustic Focusing Cytometer (Life Technologies Applied Biosystems, Vic, Australia) with an air-cooled argon-ion laser operating at 488 nm. Data was acquired using the Attune® Software (version 1.2.5, Life Technologies Applied Biosystems) and analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA). Putative contamination with cytosolic compounds was screened and the elimination of contamination by nuclei doublets, partial nuclei, nuclei with associated cytoplasm and other debris was performed as described previously (Loureiro et al. 2007a,b). Around 5,000 nuclei were analyzed per sample. The ploidy levels and putative occurrence of aneuploidy or polyploidy was determined by analyzing the G_0/ G_1 peaks position and/or appearance of new G_0/G_1 peaks.

Data analysis

The survival rate in each cryopreservation experiments for each ECL was estimated as the percentage of replicates that showed proliferation on the 4^{th} subculture after being subjected to freezing and thawing treatments. Each replicate with at least 0.25 cm^3 of new embryogenic tissue was considered as showing proliferation.

The samples were weighed asseptically at day 0 (beginning of the first subculture), and at the end of the fourth subcultures (day i). Thereafter the regrowth rate (FWi) was determined as follow:

$$FWi = \frac{weight\ at\ day\ i - weight\ at\ day\ 0}{weight\ at\ day\ 0}$$

The tissue dispersion index (%DI), which gives information about the higher or lower survival of cells restored along the plate surface, was also calculated as based on the surface of the regrowing area, corresponding to 0, 25, 50, 75 and 100% surface, which showed recovered embryogenic cells.

Data were analyzed by a one-way analysis of variance (ANOVA) and when necessary data were transformed to achieve normality and equality of variance. When these criteria were not satisfied even with transformed data, the non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks was performed. The post hoc analysis was evaluated by Dunn's, Holm-Sidak or Student-Newman-Keuls method. The significance level was 0.05. All statistical analysis was performed using SigmaPlot for Windows, version 11.0.

Results

In order to establish a protocol to cryopreserve and recover embryogenic cultures from the tropical hybrid, different variable were evaluated, including pre-cooling period, the type of cryoprotectants, carbohydrates and DMSO based-cryoprotectants, and the freezing procedures. The effect of each variable on the survival rate (%) is shown in Table 1. Throughout all experiments the contamination rate was less than 1%.

Table 1 - Effect of treatment on the survival rate of several ECLs after thawing. Four replicates were used for each treatment per each ECL, and results were recorded after the fourth subculture.

Experiment	Treatment	ECL	Survival rate (%)
Pre-cooling storage	4 h	C.19	50
		B.47	100
		D.42	0
	24 h	C.19	100
		B.47	100
		D.42	100
DMSO based solutions	DMSO	C.19	50
		B.47	100
		D.42	0
		E.52	0
	PSD	C.19	100
		B.47	100
		D.42	100
		E.52	0
Cryoprotective carbohydrates	Sucrose	B.58	100
		B.73	25
		A.39	100
		A.41	0
		C.32	25
	Maltose	B.58	100
		B.73	25
		A.39	50
		A.41	50
		C.32	50
Cryopreservation procedures	CSF	B.58	100
		B.73	25
		A.39	100
		A.41	0
		C.32	25
	SSF I	B.58	0
		B.73	67
		A.39	50
		A.41	0
		C.32	0
	SSF II	B.58	75
		B.73	75
		A.39	67
		A.41	50
		C.32	25

Effect of pre-cooling storage

The pre-cooling period of 24 hours at -80ºC, before the immersion in liquid nitrogen gave better results than a pre-cooling of only 4 hours. Twenty-four hours of pre-cooling allowed the recovery of all ECL tested with 100% survival (Table 1) and higher values of regrowth rate. For dispersion index significative higher values were obtained (Figure 1).

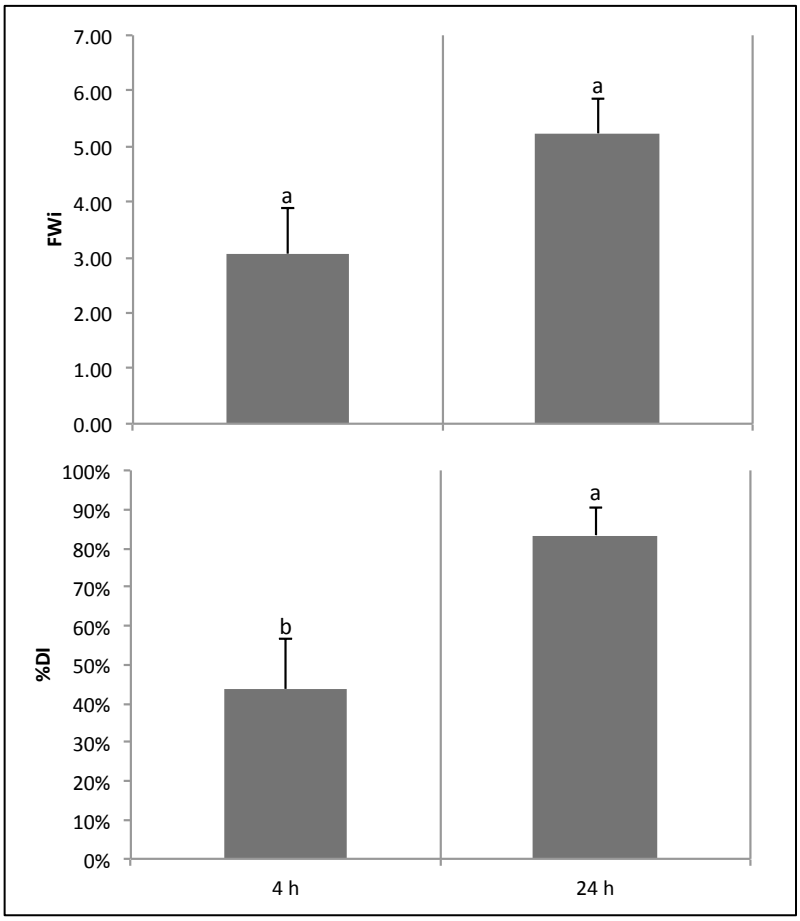

Figure 1 - Regrowth rates (FWi) and dispersion index (%DI) after different pre-cooling treatments before freezing in liquid nitrogen after 4 subcultures (8 weeks) from thawing (3 ECLs with 4 replicates per each genotype). Groups marked with different letters have significant differences, according to the Dunn's Method ($p \leq 0.05$).

Effect of DMSO based cryoprotectants

Three of the lines tested were successfully cryopreserved using the PSD solution (combines DMSO with PEG and sucrose at the same final concentration (5%) for each component), showing 100% of survival rate (Table 1). On the other hand, using DMSO alone as cryoprotectant, recovery was obtained just on two ECLs and with a lower survival rate for the line C.19 (Table 1). Regarding to the regrowth rate (FWi) and dispersion index

(Figure 2), PSD solution also stands out since the group treated with this cryoprotectant shows higher values for both parameters than the one treated with just DMSO. However, significant diferences were only obtained in the dispersion index.

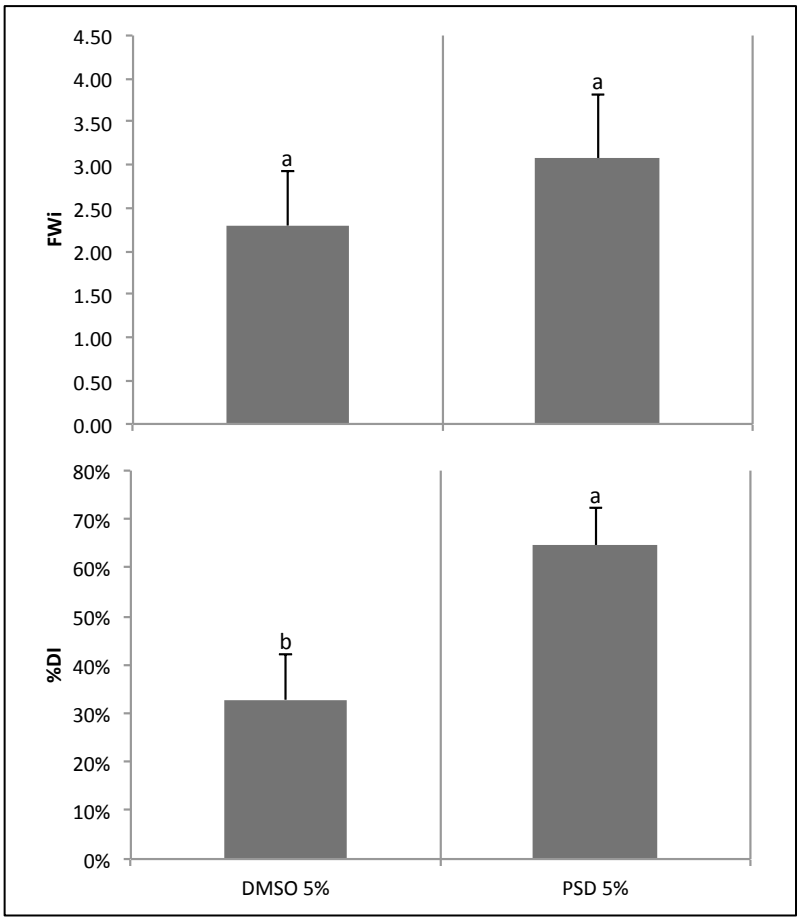

Figure 2 - Regrowth rates (FWi) and dispersion index (%DI) after pretreatment with 5% DMSO or 5% PSD after 4 subcultures (8 weeks) from thawing (three ECLs with 4 replicates per each genotype). Groups marked with different letters have significant differences, according to the Tukey's Test ($p \leq 0.05$).

Effect of carbohydrate preculture

The use of two different cryoprotective carbohydrates, sucrose *versus* maltose, during the pretreatment period were compared on five ECLs from three different families. The maltose experiment leads to the recover of all ECLs tested, with survival rates between 25 and 100%. The pretreatment with sucrose led to the recovery of only 80% of the ECLs tested (Table 1). However, regarding the regrowth rates (FWi) and dispersion index (%DI) after 4th subcultures, no diferences were obtained between the two conditions (Figure 3).

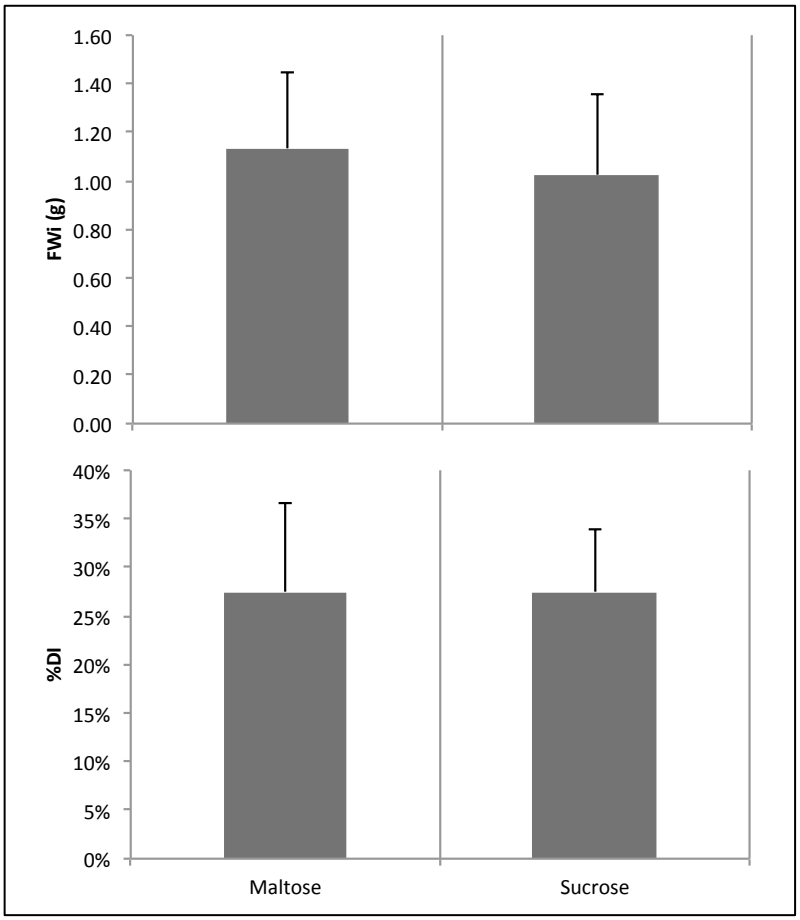

Figure 3 - Regrowth rates (FWi) and dispersion index (%DI) after pretreatment with Sucrose or Maltose (5 lines with 4 replicates per each genotypes) after 4 subcultures (8 weeks) from thawing. Data were analyzed by a Kruskal-Wallis, One Way ANOVA on Ranks but no significant differences were found.

Comparison of cryopreservation procedures

A different procedure was tested, the short slow freezing method (SSF), in which the carbohydrate pretreatment was reduced (shortened) for only 1 day. The pre-cooling time, 24 hours *versus* 4 hours, was also analyzed (SSF I and SSF II). The classical slow freezing method (CSF) was used as a control.

SSF II was the only procedure that allowed the recovery of the five ECLs tested. However it was for the CSF procedure that survival rates of 100% was reached in two of the ECLs tested. With the SSF I procedure only two lines were recovery (Table 1). This later procedure also showed lower values of regrowth rate and dispersion index (Figure 4). These data reinforce the results in which 4 hours of pre-cooling are not sufficient to allow efficient recovery of cryopreserved cells. No significant differences were found between the SSF II and the CSF (control) (Figure 4).

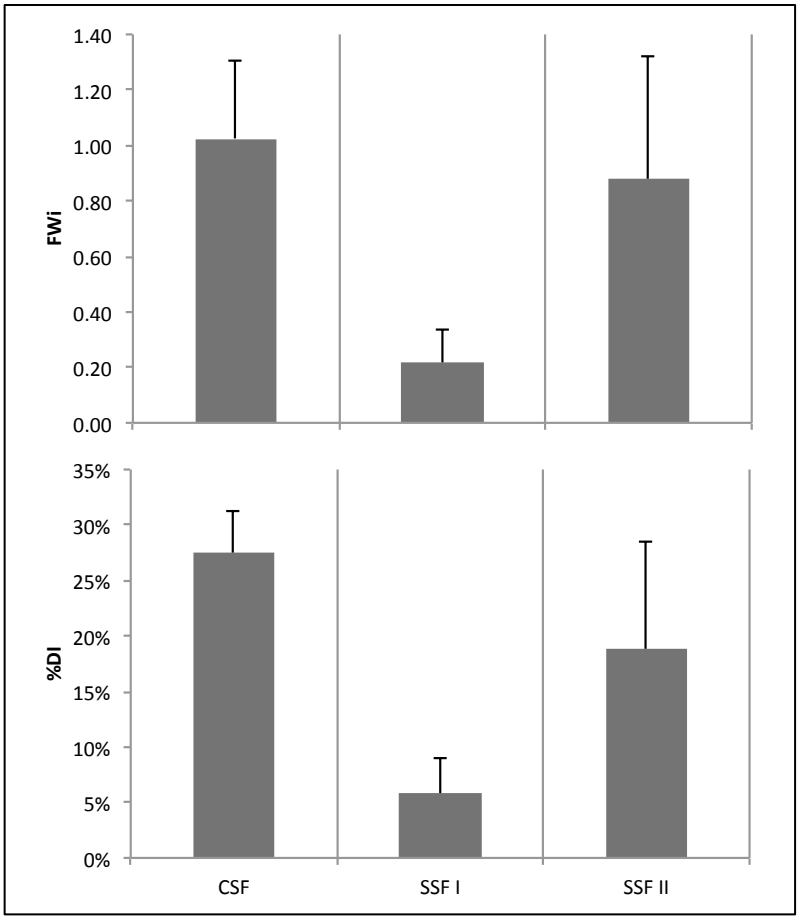

Figure 4 - Regrowth rates (FWi) and dispersion index (%DI) after three different pretreatment procedures CSF (Classical Slow Freezing method), SSF I (Short Slow Freezing method I), SSF II (Short Slow Freezing method II) after 4 subcultures (8 weeks) from thawing (five ECLs with 4 replicates per each genotype). Statistical treatment was performed by Kruskal-Wallis, One Way ANOVA on Ranks, but no significant differences were found.

Embryogenic competence evaluation and maturation ability

Embryogenic competence of ECLs cryopreserved by the CSF procedure was analyzed by monitoring the presence of embryogenic structures by light microscopy. At the same time, the maturation ability of the recovered embryogenic tissue from liquid nitrogen was evaluated.

Microscopic observation showed that EM recovered after thawing developed proembryos structures (Figure 5E to H), identical to the aspect of EM non-cryopreserved (Figure 5A to D).

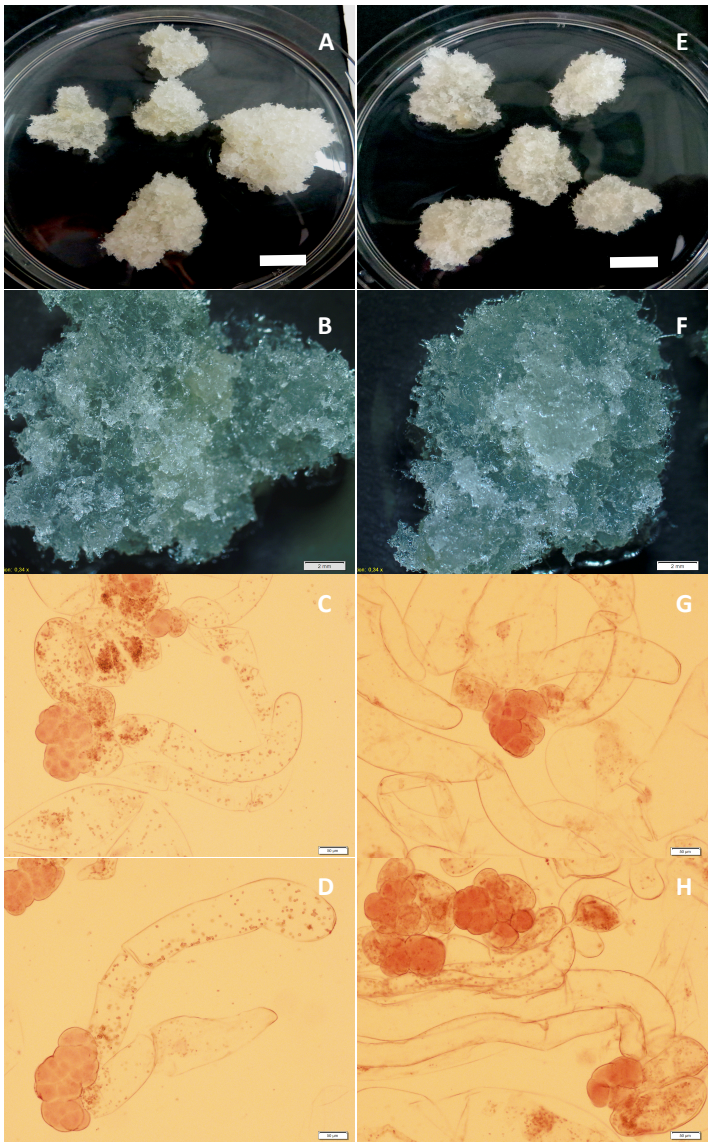

Figure 5 – Cryo and non-cryopreserved embryogenic tissues from C.5.14 of the hybrid *Pinus elliotti* var. *elliotti x Pinus caribaea* var. *hondurensis*. A and B – ECL non-cryopreserved on proliferation for about 9 months (A - bar = 1cm; B – bar = 2mm); C and D – Proembryos with long suspensors observed in the non-cryopreserved ECL stained with acetocarmine, bar = 50 µm; E and F – Recovery cryopreserved ECL in proliferation after thawing for about two months (E - bar = 1cm; F – bar = 2mm); G and H – Proembryos with long suspensors observed in the recovery cryopreserved ECL stained with acetocarmine, bar = 50 µm.

The maturation experiment using non-cryopreseved EM with 3 and 15 months in proliferation medium allowed the observation of a sharp decrease in the maturation rate and in the number of mature *Se* per gram of fresh weight of embryogenic masses with one year apart. After 3 months in proliferation, in B.47 ECL was achieved 368 mature *Se* per gram fresh weight, whereas a year later only about 45 mature *Se* was obtained. Moreover, it was identified 3 others genotypes (A.38, B.36 and B.58) that apparently lost their maturation capacity, after 15 months in proliferation (Table 2). In the B.36 ECL, the ability

to produce mature *Se* was reestablished in the recovered masses. It was also observed in B.47 and B.59 ELCs, a higher number of mature *Se* in the cryopreserved masses when compared with the non-cryopreserved masses, with the same age (Table 2).

The embryos produced from the recovered embryogenic masses after cryopreservation (Figure 6A) were converted into plantlets, and the emblings produced exhibit a good development of both root system and aerial part, similar to the emblings that came from non-cryopreserved tissue (Figure 6B and C).

Table 2 - Number of mature somatic embryos (*Se*) per gram of fresh weight of embryogenic tissue (Means ± SE. $n \geq 3$) and maturation rates for non-cryopreserved masses with 3 and 15 months in culture after initiation and cryopreserved masses with 15 months after proliferation. The statistic treatment was made by ECL, conditions marked with * have significant differences according to the Holm-Sidak Method ($p \leq 0.05$).

	Non-cryopreserved masses for 3 months in proliferation		**Non-cryopreserved masses for 15 months in proliferation**		**Cryopreserved masses for 15 months in proliferation**	
ECL	Nº Mature SE / g FW	Maturation (%)	Nº Mature SE / g FW	Maturation (%)	Nº Mature SE / g FW	Maturation (%)
A.35	164.18 ± 53.81*	100.00	17.91 ± 14.47	40.00	8.96 ± 5.97	40.00
A.38	378.11 ± 9.28	100.00	0.00	0.00	0.00	0.00
B.36	9.95 ± 9.95	33.33	0.00	0.00	2.99 ± 2.99	20.00
B.47	368.16 ± 49.00*	100.00	44.78 ± 16.35	80.00	98.51 ± 36.13	80.00
B.58	44.78 ± 17.23	100.00	0.00	0.00	0.00	0.00
B.59	119.40 ± 31.07	100.00	17.91 ± 5.58	80.00	92.54 ± 32.49	100.00
B.70	4.98 ± 0.33	33.33	0.00	0.00	0.00	0.00
E.52	164.18 ± 65.06*	100.00	2.99 ± 2.99	20.00	0.00	0.00

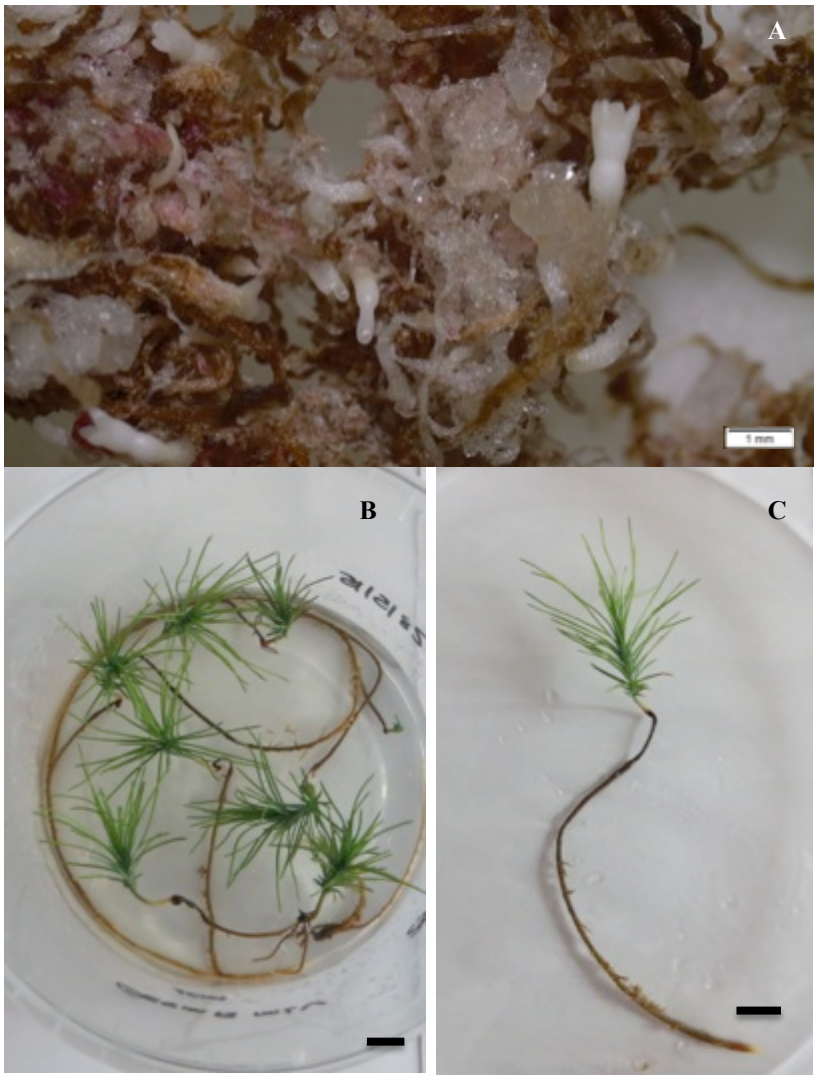

Figure 6 – Plants conversion of the hybrid *Pinus elliotti* var. *elliotti x Pinus caribaea* var. *hondurensis* mature *Se* produced by the recovered B.59 ECL after cryopreservation. A – ECL at the end of the maturation phase with mature somatic embryos, bar = 1 mm. B and C – Emblings produced by SE of the recovered ECL, bar = 1cm.

Genetic stability analysis

EM genetic stability after cryopreservation was evaluated by flow cytometry in comparison with proliferating EM of the same age but not cryopreserved. The mean values of nuclear DNA fluorescence index (DNA index = $2C_{PE}/2C_{Vicia\ faba}$) obtained for the cryopreserved EM (DI = 1.81 for E.81.14 and 1.87 for C.5.14) were similar ($p > 0.05$) to the ones obtained for the non-cryopreserved tissue (DNA index = 1.82 for E.81.14 and 1.85 for C.5.14) (Table 3) leading to similar DNA contents. The cell cycle analysis also showed no significant differences in the cell cycle phases, between the two groups of EM (Figure 7 A and B).

Table 3 – DNA index (Means ± standard deviation (SD); $n \geq 5$), Nuclear DNA content (Means ± standard deviation (SD); $n \geq 5$) and the mean coefficient of variation (%CV) of the hybrid embryogenic cell lines (ECL) E.81.14 and C.5.14, cryopreserved (Cryo) and non-cryopreserved (Non). The statistic treatment was made per each ECL, but no significant variation was found ($p \leq 0.05$).

	Cryopreserved	E.81.14	C.5.14
DNA index	Non	1.82 ± 0.02	1.85 ± 0.02
	Cryo	1.81 ± 0.02	1.87 ± 0.02
Nuclear DNA content (pg/2C)	Non	49.04 ± 0.44	49.87 ± 0.59
	Cryo	48.62 ± 0.61	50.25 ± 0.67
CV (%)	Non	5.02	5.24
	Cryo	4.62	5.33

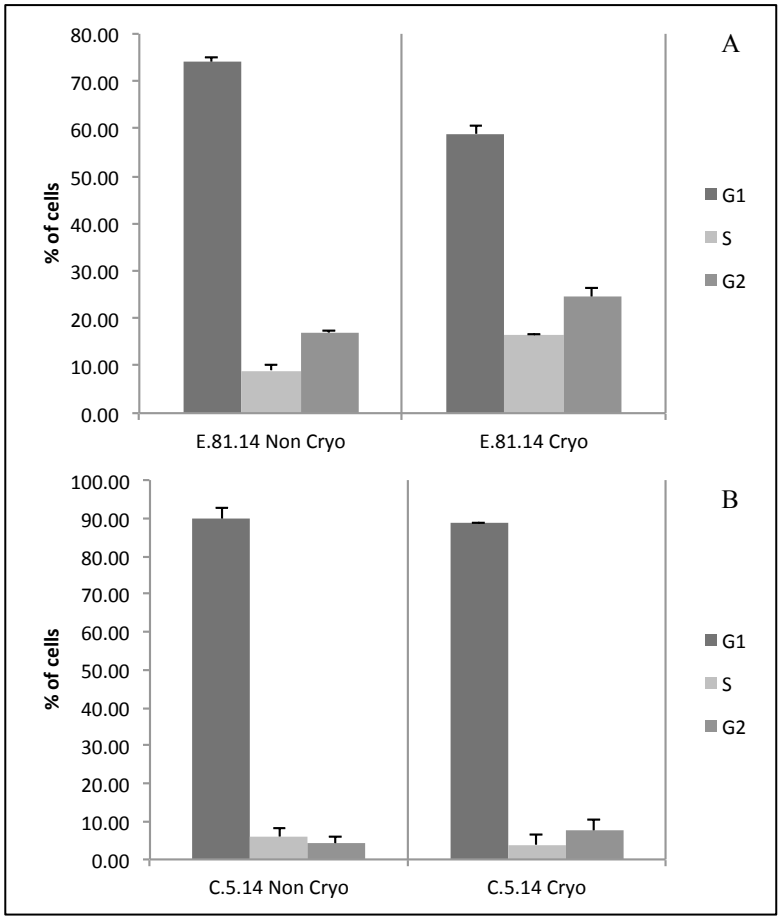

Figure 7 - Cell cycle dinamics for two ECLs (A- E.81.14 and B- C.5.14) cryo and non-cryopreserved. The statistic treatment was made per each ECL, but no significant variation was found ($p \leq 0.05$).

Discussion

In the present study, we report the establishment of a successful cryopreservation procedure for EM of the interspecific hybrid *P. elliottii* var. *elliottii x P. caribaea* var. *hondurensis*. Also, the EM viability and the embryo maturation capacity were evaluated, with success, in different genotypes recovered from the LN immersion. Several pretreatment conditions were tested on nine ECLs to access to the best conditions for slow freezing of the hybrid ECLs. All tested genotypes (100%) survived to the cryopreservation procedure in at least one condition tested, although differences in regrowth frequencies, after thawing, were observed for individual ECLs and treatments (ranging from 25 to 100%).

The ultra-low temperatures can induce cell damages during freezing process, by the formation of large ice crystals inside the cells and by the toxic solution effect as a result of dehydration. Penetrant and non-penetrant cryoprotectants can be used in order to minimize these freezing damages, by depressing both the freezing point and the supercooling of water (Chawla 2002). DMSO is one of the most used penetrant cryoprotectant in embryogenic cultures of conifers (Laine et al. 1992; Cyr et al. 1994; Ford et al. 2000a; Hargreaves et al. 2002; Mathur et al. 2003; Salaj et al. 2007; 2011; Ma et al. 2012). The choice of a cryoprotective agent and respectively concentration is dependent upon the type of cell to be preserved. In our study the use of a PSD solution (with PEG 4000, sucrose and DMSO) as a cryoprotectant, improved the EM recovery, when compared to DMSO alone. For the first one, 75% of the cryopreserved ECLs were recovered with 100% survival. These results are in accordance with those of Marum et al. (2004) and Álvarez et al. (2012) which concluded that for ECLs of *P. pinaster*, the cryopreservation protocol is significantly more efficient using PSD instead of pure DMSO. Haggman et al. (1998) also described the beneficial effect of a similar cryoprotectant solution with PEG, glucose and DMSO (PGD) in EM of *P. sylvestris*, reinforcing the idea that a mixture of cryoprotectants is often more efficient, than only one component at the same total osmolarity (Engelmann 1991).

Prior exposure to the based-DMSO cryoprotectants, it is usual to pretreat the cells with carbohydrate agents such as sugars or sugar alcohols. In our work sucrose and maltose were both evaluated, with similar results regarding regrowth rates. Sucrose is one of the most common agent used on pretreatments of *Pinus* embryogenic cultures, such as *P.*

caribaea (Laine et al. 1992), *P. sylvestris* (Häggman et al. 1998) and *P. nigra* (Salaj et al. 2007). On the other hand, maltose improved recovery of cryopreserved EMs in *P. pinaster* compared to sucrose, sorbitol and glucose (Marum et al. 2004). We demonstrate here for the hybrid EMs that, similarly to *P. nigra* (Salaj et al. 2011), maltose can be used as an alternative sugar cryoprotectant.

The slow freezing procedure is characterized by a slow cooling step to a pre-freezing temperature (around -40ºC) followed by rapid immersion in liquid nitrogen (Engelmann 2000). Little attention has been given to the importance of the duration of this slow cooling period. However it is critical because an excessive period can be detrimental to cell survival, increasing intracellular solute concentration (Mazur 1984). The freezing containers usually used in cryopreservation allow a decreasing temperature rate at -1ºC/min. In the present work, we conclude that a period of 24h during cooling is more advantageous for the ECLs recovery, when compared to the storage at -80ºC, for 4h in the freezing container CoolCell, before plunging samples directly into liquid nitrogen. In *P. pinaster*, 80 min in a freezing container at -80ºC is sufficient to reach the desired temperature of -40ºC (Álvarez et al. 2012). According to the authors, the regrowth rate of the cryopreserved EM was significantly lower when the samples were kept during 24h than in 80 min, in contrast with our results. The decreasing temperature at a slow cooling rate enables the formation of ice crystals in the extracellular solution and the removal of water from the intracellular compartments (Martinez-Montero & Harding 2015).

From the point of view of industrial application, the reduction of operating time of cryopreservation pretreatments is crucial to improve the process efficiency. These pre-treatments can vary from a few minutes (Salaj et al. 2007) to several hours reaching 3 days for the classical protocol already tested in other conifer species (Häggman et al. 1998; Marum et al. 2004; Latutrie & Aronen 2013). The shorter protocol SSFII tested in this study, allowed the recovery of 100% of the ECLs tested while with the CSF procedure only 80% regrowth was achieved after thawing. Based on our results we suggest that the shorter protocol can be an alternative for the EM cryopreservation. Similar results were also obtained in *P. pinaster* embryogenic cultures (Laine et al. 1992; Álvarez et al. 2012). More work will be performed to ascertain the applicability of the SSFII to higher number of genotypes. In the same perspective of reducing time consumed with the

cryopreservation procedure, we also tried to use a vitrification-based technique to cryopreserve our samples, but no regrowth was obtained (data not shown).

From the maturation experiments with non-cryopreserved EM in our work, it was observed a significant decreasing of mature *Se* differentiation, after one year in culture. The lost of maturation ability with increasing number of subcultures of EM has been described in some species of conifers, as is the case of *P. caribaea* (David et al. 1995), *P. pinaster* (Breton et al. 2006; Klimaszewska et al. 2009), *P. nigra* (Salajova & Salaj 2005), *P. thunbergii* and *P. densiflora* (Taniguchi 2001). Our results underline the need to cryopreserve the EMs as soon as possible to avoid the loss of embryogenic capacity. We also observed in these hybrid EMs that the cryopreservation procedure did not negatively affect the embryogenic ability to produce mature *Se*. The embryogenic ability to differentiate cotyledonary embryos from the cryopreserved and recovered EM was also maintained in other *Pinus* species (David et al. 1995; Marum et al. 2004; Malabadi & Nataraja 2006; Salaj et al. 2007; Álvarez et al. 2012; Latutrie & Aronen 2013). Three different recovered ECLs apparently presented higher maturation rates compared with non-cryopreserved EMs. Similar result was also observed on *Pseudotsuga menziesii* EM after cryopreservation and the authors have suggested that the beneficial effect of cryogenic storage on embryogenic capacity is related to the elimination of non-embryogenic cells from the cultures (as reviewed by Gupta et al. 1995). The authors also mentioned that this outcome can result from the increased synchrony of development from embryo heads, which are the only tissues surviving immersion in liquid nitrogen when cryopreserved using DMSO as a cryoprotective substance.

The assessment of genetic stability of micropropagated or cryopreserved tissues of forest species can be performed with a variety of techniques, including phenotypic/morphological observations, biochemical and molecular data (e.g. Harding 2004; Lopes et al. 2006), and more recently with flow cytometric analyzes that provide information on cultures/regenerants' genetic true-to-typeness (Fernandes et al. 2008; Marum et al. 2009; San José et al. 2015). However, scarce reports are available regarding the use of FCM to assess the genetic fidelity of micropropagated conifers (e.g. Marum et al. 2009). The current study is the first report regarding FCM use in assessing ploidy genetic stability of *Pinus* EM tissues after cryopreservation. It is generally accepted that cryopreservation blocks cell metabolism and prevents somaclonal variations caused by successive

subcultures (Engelmann 2004), which explains the little attention that has been given to study the genetic stability of the regenerated material after cryopreservation. However, the effects of cryoinjury upon the genome of a plant remain unknown, leading to the need to evaluate the genetic stability of the cryopreserved material before routinely using this technique for long-term conservation of plant germplasm (Harding 2004; Engelmann 2004). According to Park and co-workers (1998) the morphology of *Picea abies* clones remained unaffected by cryopreservation. Similar morphology was also observed in *Pseudotsuga menziesii* (Gupta et al. 1995) and *P. caribaea* (Laine et al. 1992) cryo and non-cryopreserved somatic embryo plants. In the present study the morphology of the cryo and non-cryopreserved embryogenic cultures and plantlets of the hybrid have also the same morphology. The FCM results suggest no major changes in the genetic fidelity of the hybrid embryogenic cultures due to cryopreservation process. Also the cell cycle analysis showed that the cryopreservation protocol did not affect cell cycle dynamics of the hybrid ECLs. FCM is an accurate method for genome size determination and ploidy level determination (Loureiro et al. 2007a-c; Brito et al. 2008), that was integrated in breeding programs of woody species to assess genetic stability in *in vitro* protocols (Conde et al. 2004; Loureiro et al. 2007c; Fernandes et al. 2008; Marum et al. 2009). Its use was also valuable in assessing cryopreserved somatic embryos of cork oak (Fernandes et al. 2008) and *Alnus glutinosa* (San José et al. 2015).

Using randomly amplified polymorphic DNA (RAPD), no genetic instability was observed in plantlets regenerated from cryopreserved apices and EM of *Prunus* (Helliot et al. 2002) and *Dioscorea bulbifera* (Dixit et al. 2003), respectively. After a slow freezing cryopreservation procedure, using PGD as cryoprotectant solution, Häggman et al. (1998) also showed no genetic variation, by RAPDs analysis, on EM of *P. sylvestris*. On the other hand, Salaj et al. (2011), detected higher genetic instability in non-frozen embryogenic cultures treated with 7.5% of DMSO of *P. nigra,* than in cryostored samples. Therefore, the genetic instability was not due to freezing itself but to the pretreatment (Salaj et al. 2011). Similar results were reported for non-frozen EM of *Abies cephalonica* treated with 5% DMSO (Aronen et al. 1999). It has been postulated that DMSO can cause genetic alterations (Vannini & Poli 1983) due to an effect on membrane permeability and function by interfering with enzyme systems and by altering O_2 uptake (Friend & Freedman 1978). Also, DMSO may interfere with the thermo stability of chromosome structure, inhibiting

DNA synthesis, altering the secondary structure of DNA and RNA and causing scission in DNA and alterations in folded genomes (Aronen et al. 1999). It was therefore suggested that although cryostorage does not remove the mutagenic potential of DMSO, it may eliminate a high proportion of cells bearing genetic changes (Aronen et al. 1999; Salaj et al. 2011). Aronen and co-workers (1999) also used PGD treatments on their study, concluding that only background level of genetic variation was found in the *Abies* ECLs, indicating that the other compounds of the cryoprotectant mixture used (polyethylene glycol and glucose) can probably diminish the detrimental effects of DMSO. In our work we also selected a mixture of cryoprotectants over the use of pure DMSO, and no major genetic variation was found, which is in accordance with the findings of Häggman et al. (1998) and Aronen et al. (1999).

Conclusion

In conclusion, we demonstrate here a robust protocol of cryopreservation of EMs of the highly important hybrid *Pinus elliottii* var. *elliottii x Pinus caribaea* var. *hondurensis*. The efficient protocol included slow freezing cryopreservation approach leading to efficient regrowth of cryopreserved tissue for most of the ECLs studied. We also presented maltose as an alternative sugar cryoprotectant and conclude that the use of PSD solution instead of DMSO alone is beneficial to the process. In addition, in cryopreserved tissue, no genetic variation has been found and there was no loss of embryogenic potential when 5% PSD is used as cryoprotectant mixture.

References

Álvarez, José M., Millán Cortizo, and Ricardo J. Ordás. 2012. "Cryopreservation of Somatic Embryogenic Cultures of *Pinus pinaster*: Effects on Regrowth and Embryo Maturation." *Cryoletters* 33 (6). Cryoletters: 476–84.

Aronen, Tuija S., Jana Krajnakova, Hely M. Häggman, and Leena A. Ryynänen. 1999. "Genetic Fidelity of Cryopreserved Embryogenic Cultures of Open-Pollinated Abies Cephalonica." *Plant Science* 142 (2): 163–72. doi:10.1016/S0168-9452(98)00244-1.

Breton, David, Luc Harvengt, Jean-François Trontin, Alain Bouvet, and Jean-Michel Favre. 2006. "Long-Term Subculture Randomly Affects Morphology and Subsequent Maturation of Early Somatic Embryos in Maritime Pine." *Plant Cell, Tissue and Organ Culture* 87 (1): 95–108. doi:10.1007/s11240-006-9144-9.

Brito, Gina, João Loureiro, Tina Lopes, Eleazar Rodriguez, and Conceição Santos. 2008. "Genetic Characterisation of Olive Trees from Madeira Archipelago Using Flow Cytometry and Microsatellite Markers." *Genetic Resources and Crop Evolution* 55 (5): 657–64. doi:10.1007/s10722-007-9268-8.

Chawla, H. S. 2002. *Introduction to Plant Biotechnology*. Science Publishers.

Conde, P, J Loureiro, and C Santos. 2004. "Somatic Embryogenesis and Plant Regeneration from Leaves of Ulmus Minor Mill." *Plant Cell Reports* 22 (9): 632–39. doi:10.1007/s00299-003-0735-1.

Cyr, D R, W R Lazaroff, S M Grimes, G Quan, T D Bethune, D I Dunstan, and D R Roberts. 1994. "Cryopreservation of Interior Spruce (Picea Glauca Engelmanni Complex) Embryogenic Cultures." *Plant Cell Reports* 13 (10): 574–77. doi:10.1007/BF00234514.

David, A, E Laine, and H David. 1995. "Somatic Embryogenesis in *Pinus Caribaea*." In *Somatic Embryogenesis in Woody Plants*, 145–81. Springer.

DeVerno, L. L., Y. S. Park, J. M. Bonga, J. D. Barrett, and C. Simpson. 1999. "Somaclonal Variation in Cryopreserved Embryogenic Clones of White Spruce [Picea Glauca (Moench) Voss.]." *Plant Cell Reports* 18 (11): 948–53. doi:10.1007/s002990050689.

Dieters, Mark, and Jeremy Brawner. 2007. "Productivity of *Pinus Elliottii*, P. Caribaea and Their F1 and F2 Hybrids to 15 Years in Queensland, Australia." *Annals of Forest Science* 64 (7): 691–98. doi:10.1051/forest:2007049.

Dixit, Sonali, B B Mandal, Sangeeta Ahuja, and P S Srivastava. 2003. "Genetic Stability Assessment of Plants Regenerated from Cryopreserved Embryogenic Tissues of Dioscorea Bulbifera L. Using RAPD, Biochemical and Morphological Analysis." *Cryo Letters* 24 (2): 77–84.

Dumet, D., and E. E. Benson. 2000. "The Use of Physical and Biochemical Studies to Elucidate and Reduce Cryopreservation-Induced Damage in Hydrated/desiccated Plant Germplasm." In *Cryopreservation of Tropical Plant Germplasm*, edited by F. Engelmann and H. Takagi, 43–56. Tsukuba, Japan: International Plant Genetic Resources Institute (IPGRI).

Engelmann, F. 2000. "Importance of Cryopreservation for the Conservation of Plant Genetic Resources." In *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application. Proceedings of an International Workshop*, edited by F Engelmann and H Takagi, 8–20.

Engelmann. 2011. “Cryopreservation of Embryos: An Overview.” *Methods in Molecular Biology (Clifton, N.J.)* 710 (January): 155–84. doi:10.1007/978-1-61737-988-8_13.

Engelmann, F. 1991. “In Vitro Conservation of Tropical Plant Germplasm ? A Review.” *Euphytica* 57 (3): 227–43. doi:10.1007/BF00039669.

Engelmann, Florent. 2004. “Plant Cryopreservation: Progress and Prospects.” *In Vitro Cellular & Developmental Biology - Plant* 40 (5): 427–33. doi:10.1079/IVP2004541.

Fernandes, P., E. Rodriguez, G. Pinto, I. Roldan-Ruiz, M. De Loose, and C. Santos. 2008. “Cryopreservation of Quercus Suber Somatic Embryos by Encapsulation-Dehydration and Evaluation of Genetic Stability.” *Tree Physiology* 28 (12): 1841–50. doi:10.1093/treephys/28.12.1841.

Ford, C S., N B. Jones, and J van Staden. 2000b. “Optimization of a Working Cryopreservation Protocol for *Pinus Patula* Embryogenic Tissue.” *In Vitro Cellular & Developmental Biology - Plant* 36 (5): 366–69. doi:10.1007/s11627-000-0065-8.

Ford, C. S., N. B. Jones, and J. van Staden. 2000a. “Cryopreservation and Plant Regeneration from Somatic Embryos of *Pinus Patula*.” *Plant Cell Reports* 19 (6): 610–15. doi:10.1007/s002990050781.

Friend, C, and H A Freedman. 1978. “Effects and Possible Mechanism of Action of Dimethylsulfoxide on Friend Cell Differentiation.” *Biochemical Pharmacology* 27 (9): 1309–13.

Gupta, P K, and D J Durzan. 1985. “Shoot Multiplication from Mature Trees of Douglas-Fir (Pseudotsuga Menziesii) and Sugar Pine (*Pinus Lambertiana*).” *Plant Cell Reports* 4 (4): 177–79. doi:10.1007/BF00269282.

Gupta, P K, R Timmis, K A Timmis, W C Carlson, and E D E Welty. 1995. “Somatic Embryogenesis in Douglas-Fir (Pseudotsuga Menziesii).” In *Somatic Embryogenesis in Woody Plants*, 303–13. Springer.

Gupta, P. K., D. J. Durzan, and B.J. Finkle. 1987. “Somatic Polyembryogenesis in Embryogenic Cell Masses of Picea Abies (Norway Spruce) and *Pinus Taeda* (loblolly Pine) after Thawing from Liquid Nitrogen.” *Canadian Journal of Forest Research* 17 (9): 1130–34. doi:10.1139/x87-172.

Häggman, Hely M., Leena A. Ryynänen, Tuija S. Aronen, and Jana Krajnakova. 1998. “Cryopreservation of Embryogenic Cultures of Scots Pine.” *Plant Cell, Tissue and Organ Culture* 54 (1). Kluwer Academic Publishers: 45–53. doi:10.1023/A:1006104325426.

Harding, Keith. 2004. “Genetic Integrity of Cryopreserved Plant Cells: A Review.” *Cryo Letters* 25 (1): 3–22.

Hargreaves, C, L Grace, and D Holden. 2002. “Nurse Culture for Efficient Recovery of Cryopreserved *Pinus Radiata* D. Don Embryogenic Cell Lines.” *Plant Cell Reports* 21 (1): 40–45. doi:10.1007/s00299-002-0478-4.

Helliot, B., D. Madur, E. Dirlewanger, and M. T. Boucaud. 2002. “Evaluation of Genetic Stability in Cryopreserved Prunus.” *In Vitro Cellular & Developmental Biology - Plant* 38 (5): 493–500. doi:10.1079/IVP2002325.

Kartha, K.K., L.C. Fowke, N.L. Leung, K.L. Caswell, and I. Hakman. 1988. “Induction of Somatic Embryos and Plantlets from Cryopreserved Cell Cultures of White Spruce (Picea Glauca).” *Journal of Plant Physiology* 132 (5): 529–39. doi:10.1016/S0176-1617(88)80249-9.

Klimaszewska, K., C. Noceda, G. Pelletier, P. Label, R. Rodriguez, and M. A. Lelu-Walter. 2009.

"Biological Characterization of Young and Aged Embryogenic Cultures of *Pinus Pinaster* (Ait.)." *In Vitro Cellular & Developmental Biology - Plant* 45 (1): 20–33. doi:10.1007/s11627-008-9158-6.

Laine, E, P Bade, and A David. 1992. "Recovery of Plants from Cryopreserved Embryogenic Cell Suspensions of *Pinus Caribaea*." *Plant Cell Reports* 11 (5-6): 295–98. doi:10.1007/BF00235085.

Latutrie, Mathieu, and Tuija Aronen. 2013. "Long-Term Cryopreservation of Embryogenic *Pinus Sylvestris* Cultures." *Scandinavian Journal of Forest Research* 28 (2). Taylor & Francis Group: 103–9. doi:10.1080/02827581.2012.701325.

Litvay, J D, D C Verma, and M A Johnson. 1985. "Influence of a Loblolly Pine (*Pinus Taeda* L.). Culture Medium and Its Components on Growth and Somatic Embryogenesis of the Wild Carrot (Daucus Carota L.)." *Plant Cell Reports* 4 (6): 325–28. doi:10.1007/BF00269890.

Lopes, T, G Pinto, J Loureiro, A Costa, and C Santos. 2006. "Determination of Genetic Stability in Long-Term Somatic Embryogenic Cultures and Derived Plantlets of Cork Oak Using Microsatellite Markers." *Tree Physiology* 26: 1145–52.

Loureiro, J., A. Capelo, G. Brito, E. Rodriguez, S. Silva, G. Pinto, and C. Santos. 2007a. "Micropropagation of Juniperus Phoenicea from Adult Plant Explants and Analysis of Ploidy Stability Using Flow Cytometry." *Biologia Plantarum* 51 (1): 7–14. doi:10.1007/s10535-007-0003-2.

Loureiro, João, Eleazar Rodriguez, Jaroslav Dolizel, and Conceição Santos. 2007b. "Two New Nuclear Isolation Buffers for Plant DNA Flow Cytometry: A Test with 37 Species." *Annals of Botany* 100: 875–888. doi:10.1093/annbot/mcm152.

Loureiro, João, Eleazar Rodriguez, Armando Costa, and Conceição Santos. 2007c. "Nuclear DNA Content Estimations in Wild Olive (Olea Europaea L. Ssp. Europaea Var. Sylvestris Brot.) and Portuguese Cultivars of O. Europaea Using Flow Cytometry." *Genetic Resources and Crop Evolution* 54 (1): 21–25. doi:10.1007/s10722-006-9115-3.

Ma, X., K. Bucalo, R. O. Determann, J. M. Cruse-Sanders, and G. S. Pullman. 2012. "Somatic Embryogenesis, Plant Regeneration, and Cryopreservation for Torreya Taxifolia, a Highly Endangered Coniferous Species." *In Vitro Cellular & Developmental Biology - Plant* 48 (3): 324–34. doi:10.1007/s11627-012-9433-4.

Maeno, N, and G Hawtin. 2000. "Foreword." In *Cryopreservation of Tropical Plant Germplasm*, edited by F Engelmann and H Takagi.

Malabadi, Ravindra B., and K. Nataraja. 2006. "Cryopreservation and Plant Regeneration via Somatic Embryogenesis Using Shoot Apical Domes of Mature *Pinus Roxburghii* Sarg, Trees." *In Vitro Cellular & Developmental Biology - Plant* 42 (2): 152–59. doi:10.1079/IVP2005731.

Martinez-Montero, ME, and K Harding. 2015. "Cryobionomics: Evaluating the Concept in Plant Cryopreservation." In *PlantOmics: The Omics of Plant Science*, edited by Debmalya Barh, Muhammad Sarwar Khan, and Eric Davies, 655–82. New Delhi: Springer India. doi:10.1007/978-81-322-2172-2.

Marum, Liliana, Catarina Estêvão, M. Margarida Oliveira, Sara Amâncio, Lucília Rodrigues, and Célia Miguel. 2004. "Recovery of Cryopreserved Embryogenic Cultures of Maritime Pine - Effect of Cryoprotectant and Suspension Density." *Cryoletters* 25 (5). Cryoletters: 363–74.

Marum, Liliana, João Loureiro, Eleazar Rodriguez, Conceição Santos, M. Margarida Oliveira, and Célia Miguel. 2009. "Flow Cytometric and Morphological Analyzes of *Pinus Pinaster*

Somatic Embryogenesis." *Journal of Biotechnology* 143 (4): 288–95. doi:10.1016/j.jbiotec.2009.08.001.

Mathur, G., V.A. Alkutkar, and R.S. Nadgauda. 2003. "Cryopreservation of Embryogenic Culture of *Pinus Roxburghii*." *Biologia Plantarum* 46 (2). Kluwer Academic Publishers: 205–10. doi:10.1023/A:1022894409408.

Mazur, P. 1984. "Freezing of Living Cells: Mechanisms and Implications." *The American Journal of Physiology* 247 (3 Pt 1): C125–42.

Murashige, T, and F Skoog. 1962. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures." *Physiologia Plantarum* 15 (3): 473–97. doi:10.1111/j.1399-3054.1962.tb08052.x.

Nikles, DG. 2000. "Experience with Some *Pinus* Hybrids in Queensland, Australia." In *Proceedings of QFRI/CRC-SPF Symposium: Hybrid Breeding and Genetics of Forest Trees*, edited by HS Dungey, MJ Dieters, and DG Nikles, 27–43. Noosa, Queensland, Australia: Department of Primary Industries, Brisbane.

Nørgaard, Jens Viktor, Valerie Duran, Øystein Johnsen, Peter Krogstrup, Snorri Baldursson, and Sara Von Arnold. 1993. "Variations in Cryotolerance of Embryogenic Picea Abies Cell Lines and the Association to Genetic, Morphological, and Physiological Factors." *Canadian Journal of Forest Research* 23 (12): 2560–67. doi:10.1139/x93-317.

Park, Y. S., J. D. Barrett, and J. M. Bonga. 1998. "Application of Somatic Embryogenesis in High-Value Clonal Forestry: Deployment, Genetic Control, and Stability of Cryopreserved Clones." *In Vitro Cellular & Developmental Biology - Plant* 34 (3): 231–39. doi:10.1007/BF02822713.

Pinto, G, S Silva, Y Park, L Neves, C Araújo, and C Santos. 2008. "Factors Influencing Somatic Embryogenesis Induction in Eucalyptus Globulus Labill.: Basal Medium and Anti-Browning Agents." *Plant Cell, Tissue and Organ Culture* 95 (1): 79–88. doi:10.1007/s11240-008-9418-5.

Pinto, G, H Valentim, A Costa, S Castro, and C Santos. 2002. "Somatic Embryogenesis in Leaf Callus from a Mature Quercus Suber L. Tree." *In Vitro Cellular & Developmental Biology - Plant* 38 (6): 569–72. doi:10.1079/IVP2002352.

Reinhoud, PJ, F van Iren, and JW Kijne. 2000. "Cryopreservation of Undifferentiated Plant Cells." In *Cryopreservation of Tropical Plant Germplasm*, 91–102.

Salaj, T., I. Matušíková, L. Fráterová, B. Piršelová, and J. Salaj. 2011. "Regrowth of Embryogenic Tissues of *Pinus Nigra* Following Cryopreservation." *Plant Cell, Tissue and Organ Culture (PCTOC)* 106 (1): 55–61. doi:10.1007/s11240-010-9893-3.

Salaj, Terezia, Bart Panis, Rony Swennen, and Jan Salaj. 2007. "Cryopreservation of Embryogenic Tissues of *Pinus Nigra* Arn. by a Slow Freezing Method." *Cryoletters* 28 (2). Cryoletters: 69–76.

Salajova, T., and J. Salaj. 2005. "Somatic Embryogenesis in *Pinus Nigra*: Embryogenic Tissue Initiation, Maturation and Regeneration Ability of Established Cell Lines." *Biologia Plantarum* 49 (3): 333–39. doi:10.1007/s10535-005-0003-z.

San José, M. C., L. V. Janeiro, and E. Corredoira. 2015. "Simple Strategy for the in Vitro Conservation of Alnus Glutinosa (L.) Gaertn. Germplasm." *Trees* 29 (2). Springer Berlin Heidelberg: 539–49. doi:10.1007/s00468-014-1133-8.

Taniguchi, Toru. 2001. "Plant Regeneration from Somatic Embryos in *Pinus Thunbergii* (Japanese Black Pine) and *Pinus Densiflora* (Japanese Red Pine)." In *Progress in Biotechnology*,

18:319–24. Progress in Biotechnology. Elsevier. doi:10.1016/S0921-0423(01)80088-5.

Vannini, G. L., and F. Poli. 1983. "Binucleation and Abnormal Chromosome Distribution inEuglena Gracilis Cells Treated with Dimethyl Sulfoxide." *Protoplasma* 114-114 (1-2): 62–66. doi:10.1007/BF01279869.

von Arnold, Sara, Izabela Sabala, Peter Bozhkov, Julia Dyachok, and Lada Filonova. 2002. "Developmental Pathways of Somatic Embryogenesis." *Plant Cell, Tissue and Organ Culture* 69 (3). Kluwer Academic Publishers: 233–49. doi:10.1023/A:1015673200621.

CHAPTER V
CONCLUDING REMARKS

Concluding Remarks

This work contributes with significant advances for the *in vitro* propagation and preservation of slash pine and the hybrid *P. elliottii x P. caribaea*, both with high economic value. These advances are of great importance for the development of breeding programs particularly to the implementation of multi-varietal forestry (MVF).

In vitro clonal propagation can be achieved by different methods as described in Chapter I of this thesis. The work presented here uses two of the most described methodologies for *Pinus* micropropagation: axillary shoot proliferation, and somatic embryogenesis, and the results obtained clearly go beyond the current state of the art in these genotypes.

For *P. elliottii* a simple and efficient micropropagation protocol was developed starting from seedling-shoot apices and leading to the induction of axillary shoot proliferation. All the steps of the process were optimized, from disinfection and *in vitro* germination up to acclimatization, with the production of at least 3 plants per explant in each multiplication cycle, which lasts 20 to 22 weeks. The WV5 showed to be the best basal medium for the different phases of the process; 10 - 25 μM of BAP induced a higher number of shoots per explant with no need to add auxin for an efficient bud induction; a continuous exposure to IBA alone or in combination with NAA promote the satisfactory rooting of the shoots that allows a 90% acclimatization efficiency. It was also demonstrated that the plants produced by this optimized protocol showed a physiological performance similar to seed-derived plants, suggesting that the micropropagated plants achieved the acclimatization process with success. Genetic fidelity was verified by flow cytometry, by the determination of DNA content and DNA-ploidy, and no differences were found between micropropagated and seed-derived plants.

The development of an efficient micropropagation protocol that does not promote plants genetic mutations and physiological performance changes, allows not only the clonal propagation of *P. elliottii* but also its preservation ex situ by *in vitro* storage. *In vitro* storage offers the advantage of storage under secure, controlled environmental conditions and supports the spread of disease-free germplasm.

Clonal propagation of the hybrid *P. elliottii x P. caribaea* was achieved by somatic embryogenesis. To our knowledge, this was the first SE protocol described for this tropical hybrid. In the chapter III it was developed a simple protocol that can be a kick-off for large-scale *Se* production of the hybrid in study. The different stages of the SE process, from the initiation to plant regeneration, were studied and some principal conclusions can be highlighted: mLV is the most appropriate basal medium for the SE process; the PGR combination had a stronger influence on initiation and establishment rates than basal medium composition, having been selected a combination of BAP and 2,4-D for these phases; genotype has a great influence both in the initiation and maturation; the proliferation medium has a significant influence on maturation. Plantlets conversion was achieved with a success of 60 to 86%, suggesting that an efficient SE process was developed for *P. elliottii x P. caribaea.* The SE process developed also demonstrated that it does not promote large mutations, and emblings showed by FCM true-to-typeness.

Relatively to the ES process it was demonstrated in Chapter IV that, for the hybrid under study, preservation of proliferating embryogenic masses for long periods is not possible, since EM lose their embryogenic potential. This fact underscored the need to develop a cryopreservation protocol for the hybrid EM. So, a slow-freezing cryopreservation protocol was developed, showing, so far as we know for the first time, that the hybrid can be cryopreserved. Several variables were tested in order to optimize the cryopreservation process leading to the following conclusions: the use of PSD instead of DMSO alone is beneficial to the process; maltose can be an alternative to sucrose as sugar cryoprotectant; the pre-treatment period can be shortened by one day without greater prejudice for the EM reestablishment; a pre-cooling storage of 4 hours has an negative influence on EM reestablishment for both CSF and SSF methods. Finall, it was demonstrated that there was no major genetic or morphological variations, or loss of embryogenic potential in the cryopreserved tissues.

Challenges for the future

Despite the progress made over the work described in this thesis, which greatly contributed to the development of the research areas under study, when applied to *P. elliottii and P. elliottii x P. caribaea*, new studies should be conducted in order to improve the developed protocols and explore new methodologies, in particular, for preservation of mature genotypes.

The currently developed protocols clearly go beyond the current state of art in the micropropagation strategies for these genotypes. Particularly the reproducibility to different genotypes of the protocols is notable. Therefore, one future goal is the application of the methodologies developed for *P. elliottii* to the hybrid, and contrariwise. The optimization of procedures that can be applied to both *Pinus* is advantageous at the operational level when the goal is a large-scale production. Moreover, the adaptation of these protocols to adult material is challenging but necessary.

Also, with respect to the axillary shoot proliferation further studies could lead to the process optimization by the increase of the rooting rates, improving a protocol that already allows a high efficiency.

Further research on the improvement of the SE initiation rates could also be beneficial, since the main objective of these techniques is the use on multi-varietal forestry and in these programs keeping genetic variability is imperative for obtaining genetic gains at long term. Also further studies for introducing molecular techniques in assessing true to typeness would be an added value to these protocols.

Regarding to the preservation of the germplasm bank created by axillary shoot proliferation, although, till the end of the project, it has not verified disadvantages on the preservation *in vitro* of PE shoots, this maintenance is costly and susceptible to loss of material by contamination or human error. It will therefore be advantageous the investment in the development of buds cryopreservation procedures. During this project, some experiments were made using the encapsulation-dehydration technique, but without success, so, further research on this topic is needed on this area.

The application of vitrification-based methods for EM cryopreservation could simplify the cryogenic procedure since they are ultra-rapid freezing techniques. Also during this project

some attempts were made to apply this technique to EM cryopreservation, but it will be necessary to continue this research in order to obtain satisfactory results.

Although preliminary evaluations carried out during this project gives good prospects, the final evaluation of the *in vitro* propagation process will only be achieved when propagated plants physiology and productivity are evaluated in the field.